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<b>(21) International Application Number:</b> PCT/US93/05366  <b>(22) International Filing Date:</b> 4 June 1993 (04.06.93)  <b>(30) Priority data:</b> 07/894,498 4 June 1992 (04.06.92) US 07/927,200 6 August 1992 (06.08.92) US 07/992,687 17 December 1992 (17.12.92) US  <b>(60) Parent Applications or Grants</b> <b>(63) Related by Continuation</b> US 07/894,498 (CIP) Filed on 4 June 1992 (04.06.92) US 07/927,200 (CIP) Filed on 6 August 1992 (06.08.92) US 07/992,687 (CIP) Filed on 17 December 1992 (17.12.92)  <b>(71) Applicant (for all designated States except US):</b> THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 300 Lakeside Drive, 22nd Floor, Oakland, CA 94612-3550 (US).	<b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> DEBS, Robert, J. [US/US]; 12 Eton Way, Mill Valley, CA 94941 (US). ZHU, Ning [US/US]; 2137 17th Avenue, San Francisco, CA 94116 (US).  <b>(74) Agent:</b> RAE-VENTER, Barbara; Fish & Richardson, 2200 Sand Hill Road, Suite 100, Menlo Park, CA 94025 (US).  <b>(81) Designated States:</b> AT, AU, BB, BG, BR, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>	
<b>(54) Title:</b> METHODS AND COMPOSITIONS FOR <i>IN VIVO</i> GENE THERAPY  <b>(57) Abstract</b>  <p>Novel methods and compositions are provided for introducing a gene capable of modulating the genotype and phenotype into two or more tissues following systemic administration. The gene can be introduced into a mammalian host by way of an expression vector either as naked DNA or associated with lipid carriers, particularly cationic lipid carriers. Multiple individual tissues can be transfected using naked DNA. Using a DNA: lipid carrier complex, multiple tissues and cell types can be transfected. The techniques and compositions find use in the palliation or treatment of any of a variety of genetic-based disorders.</p>		

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**METHODS AND COMPOSITIONS FOR IN VIVO  
GENE THERAPY**

**INTRODUCTION**

**5   Technical Field**

The present invention relates to methods and compositions for systemic introduction of exogenous genetic material into mammalian, particularly human, cells *in vivo*.

**10   Background**

An ever-expanding array of genes for which abnormal expression is associated with life-threatening human diseases is being cloned and identified. The ability to express such cloned genes in humans will ultimately permit the prevention and/or cure of many important human diseases, diseases which now either are treated poorly or are untreatable by currently available therapies. As an example, *in vivo* expression of cholesterol-regulating genes, genes which selectively block the replication of HIV, or of tumor-suppressing genes in human patients should dramatically improve treatment of heart disease, HIV, and cancer, respectively. However, currently available gene delivery strategies have been unable to produce either a high level of or generalized transgene expression *in vivo* in a wide variety of tissues after systemic administration to a mammalian host. This inability has precluded the development of effective gene therapy for most human diseases.

30       Approaches to gene therapy include both different goals and different means of achieving those goals. The goals generally include gene replacement, gene correction and gene augmentation. In gene replacement, a mutant

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gene sequence is specifically removed from the genome and replaced with a normal, functional gene. In gene correction, a mutant gene sequence is corrected without any additional changes in the target genome. In gene  
5 augmentation, the expression of mutant genes in defective cells is modified by introducing foreign normal genetic sequences.

The means to reach the above goals used by others, have included "ex vivo" transfection of a target cell  
10 followed by introduction of the transformed cells into a suitable organ in the host mammal. Ex vivo techniques include transfection of cells in vitro with either naked DNA or DNA liposome conjugates, followed by introduction into a host organ ("ex vivo" gene therapy). The criteria  
15 for a suitable target organ or tissue include that the target organ or tissue is easily accessible, that it can be manipulated in vitro, that it is susceptible to genetic modification methods and ideally, it should contain either non-replicating cells or cycling stem  
20 cells to perpetuate a genetic correction. Further, it should be possible to reimplant the genetically modified cells into the organism in a functional and stable form. Exemplary of a target organ which meets these criteria is the mammalian bone marrow. A further criterion for  
25 ex vivo gene therapy, if for example a retroviral vector is used, is that the cells be pre-mitotic; post-mitotic cells are refractory to infection with retroviral vectors. Although this has not been reported, in some instances it may be possible to transfect cells from  
30 other than the target organ or tissue using ex vivo gene therapy if the corrective gene product can be secreted and exert the desired effect on/in the target cell following circulation in blood or other body fluids.

There are several drawbacks to ex vivo therapy;  
35 for example, if only differentiated, replicating cells

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are infected, the newly introduced gene function will be lost as those cells mature and die. *Ex vivo* approaches also can be used to transfect only a limited number of cells and cannot be used to transfect cells which are not removed first from the body. The above methods generally involve integration of new genetic material into the cell genome and thus constitute permanent changes.

Liposomes have been used effectively, particularly to introduce drugs, radiotherapeutic agents, enzymes, viruses, transcription factors and other cellular effectors into a variety of cultured cell lines and animals. The agent to be introduced is typically entrapped within the liposome, or lipid vesicle, or the agent may be bound to the outside of the vesicle. Successful clinical trials examining the effectiveness of liposome-mediated drug delivery have been completed. Several strategies have been devised to increase the effectiveness of liposome-mediated drug delivery by targeting liposomes to specific tissues and specific cell types. However, while the basic methodology for using liposome-mediated vectors is well developed, the technique has not been perfected for liposome-based transfection vectors for *in vivo* gene therapy.

*In vivo* expression of transgenes as reported by others has been restricted to injection of transgenes directly into a specific tissue, such as direct intratracheal, intramuscular or intraarterial injection of naked DNA or of DNA-cationic liposome complexes, or to *ex vivo* transfection of host cells, with subsequent reinfusion. The expression is low and generally has been limited to one tissue, typically the tissue that was injected (for example muscle); liver or lung where *iv* injection has been used; or lung where intratracheal injection has been used, and less than 1% of all cells within these tissues were transfected. In some cases,

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transfection of cells has been obtained in tissues afferent to the site of intravenous administration.

Currently available gene delivery strategies consistently have failed to produce a high level and/or  
5 generalized transgene expression *in vivo*. It therefore would be of interest to develop compositions and delivery methods for *in vivo* gene therapy that provide for a high level of transcription of the transgene and/or expression in a variety of cell and tissue types for the *in vivo*  
10 treatment, prevention, or palliation of numerous human diseases. Also of interest, is the use of gene modulation as an alternate means of gene therapy. In gene modulation, expression of an already expressed gene is increased by introducing exogenous normal genetic  
15 sequences and decreased by introducing antisense genes or gene fragments, or by introducing vectors that can produce ribozymes that can cleave specific mRNAs. Gene modulation can also be achieved by the introduction of exogenous normal genetic sequences that code for proteins  
20 that modulate the extent of gene expression, or affect the processing, assembly or secretion of gene products.

#### Relevant Literature

A large number of publications relate to *in vivo* and *ex vivo* transfection of mammals. In some cases, only  
25 transcription of a transgene has been achieved, in others, the data appear to show only a low level of expression and/or expression in a limited number of tissues or cell types. The following are examples of the publications in this area.

30 A variety of approaches for introducing functional new genetic material into cells, both *in vitro* and *in vivo* have been attempted (Friedmann, (1989) *Science*, 244:1275-1281). These approaches include integration of the gene to be expressed into modified retroviruses

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(Friedmann, (1989) *supra*; Rosenberg, (1991) *Cancer Research*, 51(18), suppl.: 5074S-5079S); integration into non-retrovirus vectors (Rosenfeld, et al., (1992) *Cell*, 68:143-155; Rosenfeld, et al., (1991) *Science*, 252:431-434); or delivery of a transgene linked to a heterologous promoter-enhancer element via liposomes (Friedmann, (1989), *supra*; Brigham, et al., (1989) *Am. J. Med. Sci.*, 298:278-281; Nabel, et al., (1990) *Science*, 249:1285-1288; Hazinski, et al., (1991) *Am. J. Resp. Cell Molec. Biol.*, 4:206-209; and Wang and Huang, (1987) *Proc. Natl. Acad. Sci. (USA)*, 84:7851-7855); coupled to ligand-specific, cation-based transport systems (Wu and Wu, (1988) *J. Biol. Chem.*, 263:14621-14624) or the use of naked DNA expression vectors (Nabel et al., (1990), *supra*); Wolff et al., (1990) *Science*, 247:1465-1468). Direct injection of transgenes into tissue produces only localized expression (Rosenfeld, (1992) *supra*); Rosenfeld et al., (1991) *supra*; Brigham et al., (1989) *supra*; Nabel, (1990) *supra*; and Hazinski et al., (1991) *supra*). The Brigham et al. group (*Am. J. Med. Sci.*, (1989) 298:278-281 and *Clinical Research*, (1991) 39 (abstract)) have reported *in vivo* transfection only of lungs of mice following either intravenous or intratracheal administration of a DNA liposome complex. An example of a review article of human gene therapy procedures is: Anderson, (1992) *Science* 256:808-813.

PCT/US90/01515 (Felgner et al.) is directed to methods for delivering a gene coding for a pharmaceutical or immunogenic polypeptide to the interior of a cell of a vertebrate *in vivo*. Expression of the transgenes is limited to the tissue of injection. PCT/US90/05993 (Brigham) is directed to a method for obtaining expression of a transgene in mammalian lung cells following either *iv* or intratracheal injection of an expression construct. PCT 89/02469 and PCT 90/06997 are

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directed to ex vivo gene therapy, which is limited to expressing a transgene in cells that can be taken out of the body such as lymphocytes. PCT 89/12109 is likewise directed to ex vivo gene therapy. PCT 90/12878 is  
5 directed to an enhancer which provides a high level of expression both in transformed cell lines and in transgenic mice using ex vivo transfection. PCT/US92/08806 is directed to particle-mediated transformation of mammalian unattached cells. EP  
10 application 91301819.8 is directed to the use of recombinant techniques to produce cystic fibrosis transmembrane conductance regulator (CFTR).

#### SUMMARY OF THE INVENTION

Methods and compositions are provided for  
15 introduction of a transgene into a plurality of mammalian tissues in vivo. The method includes the step of incorporating a transfection cassette comprising a nucleotide sequence of interest into a largely non-integrating plasmid and introducing the plasmid into a  
20 mammalian host, other than by directly introducing it into a specific tissue. The transfection cassette comprises as operably joined components, a transcriptional and initiation regulatory region, a nucleic acid sequence of interest, and a transcriptional  
25 termination regulatory region, wherein said regulatory regions are functional in the cells of a mammalian host, and wherein said nucleic acid sequence of interest is free of introns. Optionally, the transcriptional cassette is complexed with a cationic lipid carrier. The  
30 method finds use to modulate the phenotype of mammalian cells.



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DESCRIPTION OF THE DRAWINGS

Figure 1A shows photomicrographs of sections of mouse lung from a mouse injected with a transfection cassette, or Figure 1B a control mouse. The photomicrograph in 1A shows a section of mouse lung 48 hours following iv injection of pZN27:DDAB:Cholesterol expression vector-cationic lipid carrier complexes. The plasmid contained sequences for the expression of CAT (chloramphenicol acyl transferase). The lipid carrier composition was 1 to 1 molar DDAB:Cholesterol. The carrier: plasmid ratio was 5 nmoles cationic lipid to 1  $\mu$ g DNA. A dose of 100  $\mu$ g DNA was injected per mouse. This field shows alveoli and alveolar lining cells, the majority (50-70%) of which stain red positively for the presence of CAT protein when probed with anti-CAT antibody and visualized using alkaline phosphatase (indicated by arrows). The treated animals' lungs stain uniformly with diffuse involvement of alveolar and vascular endothelial cells. Airway epithelial staining is also seen indicating airway cells are also transfected. The CAT protein normally is not present in mammalian cells and therefore the presence of CAT protein in these cells indicates that they have been transfected *in vivo*. The photomicrograph in 1B shows a section of mouse lung from a control animal treated with iv-injected lipid carrier only, and probed with anti-CAT antibody. Cells do not show significant staining, although low-level background staining is detectable in some alveolar macrophages (indicated by arrows), which possess endogenous alkaline phosphatase activity.

Figure 2A shows a phase contrast micrograph of mouse T lymphocytes isolated 48 hours after intraperitoneal (ip) injection of DNA-cationic lipid carrier complexes. Lipid carriers were DDAB:DOPE, 1 to 1 molar. DNA-cationic lipid carrier complexes were 1 mg

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- pCIS-CAT with 1  $\mu$ mole cationic lipid. 1 mg DNA was injected ip. Cells were incubated with anti CAT mouse monoclonal antibody, followed by Texas red-conjugated goat anti mouse IgG. Figure 2B shows a fluorescence micrograph of the same field showing that essentially all T lymphocytes present removed from the treated animal were stained red by immunofluorescence demonstrating expression of the transfected CAT gene following ip injection of DNA/cationic lipid carrier complexes.
- Figure 2C shows a phase contrast micrograph of T lymphocytes isolated from an uninjected control mouse. Figure 2D shows a fluorescence micrograph of the same field, showing that there is no CAT gene expression in these lymphocytes from control animals.
- Figure 3A shows hematopoietic bone marrow-derived cells from a mouse treated with a DNA:lipid carrier complex DDAB:Cholesterol:pZN51 intravenously 48 hours earlier. Cells were stained for CAT protein as in Figure 1. The photomicrograph shows that approximately 70 percent of the bone marrow-derived cells, including primitive or blast cells are stained red (blast cell indicated by arrow) and are transfected in vivo. Lipid carriers were 1:1 DDAB:Cholesterol. DNA:cationic lipid ratio was 1  $\mu$ g of DNA to 5 nmoles cationic lipid. 100  $\mu$ g of DNA was injected iv into each mouse. Figure 3B shows isolated bone marrow cells from an untreated control mouse. The photomicrograph shows no red staining indicating no CAT gene expression in untreated animals.
- Figures 4A-4B show transfection of human T lymphocytes (CD4<sup>+</sup>) by CAT expression plasmid-DNA complexes in culture. The lipid carrier composition was 1:1 molar ratio DDAB:DOPE small uni-lamellar vesicles (SUV). 25  $\mu$ g of pZN27 complexed with 50 nmoles cationic lipid was added to 10 million cells in culture. Cells were incubated with anti CAT mouse monoclonal antibody,

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followed by Texas red-conjugated goat anti mouse IgG. In Figure 4A is shown a phase contrast micrograph of freshly isolated human CD4<sup>+</sup> T lymphocytes 48 hours after transfection. Figure 4B shows a fluorescence micrograph of the same field which demonstrates that essentially 100% of the T lymphocytes stain positively for CAT protein, i.e., they have been transfected.

Figure 5 shows construction of plasmid pZN20.

Figure 6A shows a restriction map of the immediate early enhancer and promoter region of HCMV (Towne) and HCMV(AD169) is shown in Figure 6C. Figure 6B shows a sequence comparison of the two HCMV promoters. The position of the NcoI site is indicated by an asterisk.

Figure 7 (panels a-f) shows electron micrographs which demonstrates that cationic lipid carrier: DNA complexes (DOTMA:DOPE:prSV-CAT) are internalized by CV-1 (African Green Monkey Kidney) cells via classical receptor-mediated endocytosis following binding to cell surface receptors. For construction of prSVCAT see Gorman et al., (1982) *Proc. Nat. Acad. Sci. USA* 79:6777-6781. Lipid carriers were 1:1 DOTMA:DOPE. 20 µg DNA were complexed with 20 nmoles cationic lipid, and added to  $2 \times 10^6$  cells. The arrows in panel (a) show particles binding to clathrin-coated pits; panel (b), the particles that have been ingested and are present in endosomes.

Figures 8A-8C show photomicrographs of a histochemical analysis of lungs from B-16 melanoma-bearing animals which received an intravenous injection of CMV-CAT cationic lipid carrier:DNA complexes. Lipid carriers were DOTAP:Cholesterol, 1 to 1 molar ratio. Cationic lipid: DNA ratio was 6 nanomoles: 1 µg DNA. The plasmid used was pZN20 (see Figure 5). 100 µg DNA was injected per mouse. The immunohistochemical analysis for CAT protein revealed intense red staining of many focal parenchymal tumors (Figure 8A indicated by arrows) and

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tumor emboli within blood vessels (Figure 8B indicated by arrows), indicating that large numbers of B16 melanoma tumor cells in the lung, as well as blood-borne metastases, are transfected after iv injection. Figure 5 8C shows that in tissue from B16 melanoma-bearing mice which did not receive an injection of DNA-lipid carrier complexes, no CAT protein is present in the surrounding normal lung or in any of the lung tumor cells.

Figure 9 shows the construction of plasmid pZN27,  
10 encoding CAT.

Figure 10A and Figure 10B show the construction of plasmid pZN46, encoding human IL-2.

Figure 11 shows the construction of plasmid pZN32, encoding human CFTR.

15 Figures 12A-12E shows photomicrographs of immunohistochemically stained (for CFTR protein) frozen sections of lung tissue from mice treated by intravenous administration of either pZN32:cationic lipid carrier complexes or lipid carrier alone (control mice). Figures 20 12A, 12C, and 12E are lung sections from mice treated with DNA lipid carrier complexes at 50X, 100X, and 250X magnification, respectively. Figures 12B and 12D are lung sections from control mice at 50X and 100X magnification show that there is no detectable CFTR 25 expression in lungs of control mice. Lipid carriers were 1 to 1 molar DDAB:Cholesterol (SUV). Lipid carrier-DNA complexes were 5 nanomoles cationic lipid to 1  $\mu$ g DNA. 100  $\mu$ g DNA was injected per mouse. Animals were sacrificed 24 hrs after injection. The figures show that 30 >70% of both airways and airway lining cells stain intensely red, indicating that they are expressing the CFTR gene.

Figure 13 shows the construction of plasmid pZN51, encoding CAT.

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Figure 14 shows the construction of plasmids pZN60, pZN61, pZN62 and pZN63, all encoding CAT. Figures 14A and 14B show the construction of intermediate plasmids pZN52, pZN54, pZN56 and pZN58. Figure 14C shows the construction of the final plasmids, pZN60 through pZN63, from the intermediates.

Figure 15 shows an autoradiograph of the thin layer chromatograph of the a CAT assay for six different plasmids injected intravenously in mice. Lanes 1-12 show the CAT activity in lung tissue; Lanes 13-24 show the CAT activity in liver tissue. Lanes 1, 2, 13, 14-pZN51; lanes 3, 4, 15, 16-pZN60; lanes 5, 6, 17, 18-pZN61; lanes 7, 8, 19, 20-pZN62; lanes 9, 10, 21, 22-pZN63; lanes 11, 12, 23, 24-pZN27. Lipid carriers were DDAB:Cholesterol (1:1). Lipid carriers-DNA complexes were 5 nmoles cationic lipid to 1 $\mu$ g DNA. 100 $\mu$ g DNA was injected per mouse. Animals were sacrificed after 48 hrs. Each lane represents a single mouse. Chromatograph runs from bottom to top of Figure as shown. As shown by the brackets in Figure 15, unreacted <sup>14</sup>C chloramphenicol only migrates a short distance from the origin of the TLC plate. The <sup>14</sup>C Acetylated chloramphenicol species migrate which result from CAT enzymatic activity further up the plate as shown by the brackets.

Figures 16A-16E show photomicrographs of frozen lung section that have been immunohistochemically stained for CAT protein. Mice were injected iv by tail vein with pZN27 (16A) which shows positive red staining for CAT is endothelial, alveolar and airway cells within the lung. In contrast, liposome complexes of the CAT gene linked to the natural CFTR promoter (pBE3.8CAT) showed CAT expression primarily in airway epithelial cells (16B). A lung section from an uninjected control animal did not show red staining, indicating no CAT expression in untransfected cells (16C). Figure 16D shows a high

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magnification photomicrograph of alveoli from a pZN27 treated mouse, and shows a high level of positive red staining for CAT gene product in both alveolar and epithelial cells. Figure 16E shows a high magnification  
5 photomicrograph of alveoli from a pBE3.8CAT injected mouse, and shows no significant CAT gene expression in either alveolar or endothelial cells. One hundred  $\mu$ g of plasmid was injected iv complexed with DDAB:Cholesterol as SUVs. Animals were sacrificed after 24 hrs.

10        Figures 16F-16K show autoradiographs of the thin layer chromatographs of CAT activity in heart (16F), lymph nodes (16G) spleen (16H), kidney (16I), lung (16J), and liver (16K) in tissue from uninjected mice (lanes 1-3), mice injected IV with pBE3.8CAT (Chou, et al., (1991)  
15 *J. Biol. Chem.*, 266:24471-24476 (lanes 4-6), or mice injected with pCIS-CAT (lanes 7-9). Lipid carriers were DDAB:Cholesterol, 1:1 SUV (1  $\mu$ g DNA to 5 nmoles cationic lipid; 100  $\mu$ g injected per mouse.

Figure 17 shows the construction of plasmid pZN84,  
20 encoding murine GM-CSF.

Figure 18 shows the construction of pZN102, encoding human CFTR.

Figure 19A shows an autoradiograph of a TLC plate showing CAT activity in animals after injection of pZN27  
25 without any lipid carrier. One milligram was injected iv twice over a 4-hour period. Mice were sacrificed 24 hrs. later. The various tissues examined are indicated under each lane.

Figure 19B shows an autoradiograph of a TLC plate  
30 showing CAT activity in an animal injected with pZN27 complexed with a lipid carrier. Lipid carrier was DDAB:Cholesterol 1 to 1 molar, 5  $\mu$ g of plasmid complexed to 1 nmole of lipid. 100  $\mu$ g was injected iv, and animals were sacrificed 24 hrs later. The various tissues  
35 examined are indicated under each lane.

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Figure 20 shows an autoradiograph of a TLC plate of injection into the right (R) ventricle of pCIS-CAT complexed to DOTMA:DOPE (1:1) lipid carriers in the indicated ratios of plasmid:lipid. Animals were

5 sacrificed 48 hrs. after the stereotactic injection of 2.5  $\mu$ g. Each pair of lanes represents a single animal. The left (L) ventricle was not injected and therefore transfection of the left ventricles demonstrates that the entire brain is being transfected.

10 Figure 21 shows human lung cancer cell line transfections. An autoradiograph of a TLC plate shows CAT activity in extracts from three different human lung cancer cell lines transfected using various cationic liposomes. The lung cancer cell lines NCI-H69, NCI-H82,

15 and NCI-H520 were plated at  $2 \times 10^6$  cells/plate and transfected in suspension. Individual plates of cells were transfected with 5  $\mu$ g of RSV-CAT (RC) alone or 5  $\mu$ g of RSV-CAT complexed to a total of 10 or 20 nmole of pure DOTMA (D), pure L-PE (L), or L-PE/CEBA (L/CA) liposomes.

20 The cells were harvested 48 h after transfection and assayed for CAT activity.

Figure 22 shows serum murine GM-CSF levels in a single goat (A) injected iv with 1 mg of GM-CSF plasmid (pZN84) complexed with DDAB:Cholesterol liposome (1  $\mu$ g

25 plasmid to 1 nmole lipid). Serum samples were collected at 0, 12, 24, 48, 72, 168, and 840 hrs. after injection. Murine GM-CSF levels were measured by ELISA using a commercial kit (Endogen).

Figure 23 show the serum murine GM-CSF levels in

30 two additional goats (B and C) injected with pZN84 as per goat A.

#### DESCRIPTION OF THE SPECIFIC EMBODIMENTS

In accordance with the subject invention, nucleic acid constructs together with methods of preparation and

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use are provided which provide for in vivo change of and/or modulation of phenotype of cells in a plurality of tissues of a mammalian host, following introduction of the constructs into the host at a dose sufficient to

5 cause transfection of tissues and cells contacted by the nucleic acid. The components of the transfection vector generally will include as operably linked components in the direction of transcription, a transcriptional initiation region, a DNA sequence of interest and a

10 transcriptional termination region, wherein the transcriptional regulatory regions are functional in the host mammal's cells that are targeted for transfection. Optionally, an intron may be included in the construct, preferably 5' to the coding sequence. Generally, the

15 construct does not become integrated into the host cell genome and is introduced into the host as part of a non-integrating plasmid where it is maintained primarily in an extrachromosomal or episomal form. The constructs can be either naked nucleic acid or nucleic acid associated

20 with a lipid carrier. By a sufficient dose is meant that which will result in a desired effect, for example prevention, palliation, and/or cure of an animal or human disease, or modulation of endogenous levels of an agent of interest ("in vivo" gene therapy). The modulation may

25 be generalized, i.e. obtained in a multiplicity of cell types or tissues, or the modulation may be selective, for example inducible and/or in only selected cell or tissue types.

Transfection of multiple tissues and cells other

30 than those solely at or afferent to the site of introduction of the nucleic acid constructs into the host is obtained and expression is at a high level and in a large number of cells and cell types. The tissues which can be transformed include the lungs, heart, liver, bone

35 marrow, spleen, lymph nodes, kidneys, thymus, skeletal



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muscle, ovary, uterus, stomach, small intestine, colon, pancreas, and brain in normal animals, as well as metastatic tumors and intravascular tumor emboli in tumor-bearing mammals. Particular cells which are

5 transfected include macrophages, alveolar type I and type II cells, hepatocytes, airway epithelial cells, vascular endothelial cells, cardiac myocytes, myeloblasts, erythroblasts, B-lymphocytes and T-lymphocytes. The route of administration typically is into a circulating

10 bodily fluid such as blood or cerebrospinal fluid, but other routes of administration also can be used. The constructs can be either naked nucleic acid or nucleic acid associated with a lipid carrier. Optionally, the lipid carrier molecule and/or construct may provide for

15 targeting and/or expression in a particular cell type or types.

The nucleic acid constructs can be prepared from nucleic acid sequences which are synthetic, naturally derived or a combination thereof. The sequences can be

20 obtained from a donor host homologous with an intended recipient host, or depending upon the nature of the nucleic acid sequence, it may be desirable to synthesize the sequence with host preferred codons. The host preferred codons may be determined from the codons of

25 highest frequency in the proteins expressed in the largest amount in the particular host of interest. Where cloning is done in a bacterial host system, it may be beneficial to change codon choice to promote stability in the bacterial host system. Where the intended use is to

30 treat disease resulting from an infectious organism, an appropriate source of sequence may be viral nucleic acid, either RNA or DNA.

Genes are high molecular weight, polyanionic molecules, for which carrier-mediated delivery is usually

35 but not always required for DNA transfection of cells

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either in culture or *in vivo*. Cationic lipid carriers such as liposomes can be used to deliver transcriptional regulatory proteins. Further, liposomes themselves (unlike viral vectors) appear to be non-immunogenic

5 *in vivo*. Liposome formulations, including those containing a cationic lipid, have been shown to be safe and well tolerated in human patients (Treat et al., (1990) *J. Natl. Cancer Instit.* 82:1706-1710). Although a wide variety of transfection techniques can produce high

10 level expression of transgenes in cultured cells, only a few such methods, including those using liposomes, are compatible with *in vivo* gene delivery. Previous attempts at using either cationic liposomes or naked DNA to obtain *in vivo* transfection have resulted in transfection of a

15 single tissue and/or transfection only of the tissue of introduction of exogenous DNA. This pattern of transfection suggests that rather than the DNA and/or liposomes being taken up by normal cellular mechanisms, cells in the tissue of introduction are damaged by the

20 introduction method and are then able to take up some of the exogenous DNA which has been introduced.

Applicants surprisingly discovered changes which provide for both transfection of a wide variety of tissues and cell types and a high-level of transgene

25 expression after systemic administration into a mammalian host. These changes include the use of DNA:cationic lipid carrier complexes, wherein the ratio of DNA to cationic lipid of the lipid carrier can significantly affect obtention of *in vivo* expression; use of a higher

30 dose of complexes than has been used previously; use of an appropriate promoter element, for example one which is both strong and constitutively active, such as the HCMV-IE1 element where it is desired to provide enhanced expression in a wide variety of cell types *in vivo*; and

35 placement of greater than 100 bp of an intron 5' to the

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coding region of the transgene or removal of the intron altogether to facilitate production of a desired gene product. Additionally, it has surprisingly been discovered that a number of tissues, including the lung, liver, spleen, kidney, lymph nodes and heart can be transformed following direct administration of high doses of naked DNA into a circulating bodily fluid, such as blood or cerebral spinal fluid. Alternatively, selective expression can be obtained in specific cell and tissue types and at a desired time (i.e. expression is inducible) by alterations in the components, particularly promoters and/or enhancers of the constructs used, or by the use of targeting moieties on the liposomes.

Most gene therapy strategies have relied on transgene insertion into retroviral or DNA virus vectors. Potential disadvantages of retroviruses, as compared to the use of naked DNA or the use of cationic lipid carriers, include the limited ability of retroviruses to mediate *in vivo* (as opposed to *ex vivo*) transgene expression; the inability of retrovirus vectors to transfect non-dividing cells; possible recombination events in replication-defective retrovirus vectors, resulting in infectious retroviruses; possible activation of oncogenes or inhibition of tumor suppressor genes due to the random insertion of the transgene into host cell genomic DNA; size limitations: less than 15 kb of DNA typically can be packaged in a retrovirus vector; and potential immunogenicity, leading to a host immune response against the vector. In addition, all *ex vivo* approaches require that the cells to be transfected be removed from the body and that they be maintained in culture for a period of time. While in culture, the cells may undergo deleterious or potentially dangerous phenotypic and/or genotypic changes. Adenoviral and other DNA viral vectors share several of the above

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potential limitations. Thus, the subject invention which does not use adenoviral or other DNA viral vectors has several advantages over existing techniques.

Additionally, the subject invention offers ease of  
5 administration and results not achievable by other means.

The constructs for use in the invention include several forms, depending upon the intended use of the construct. Thus, the constructs include vectors, transcriptional cassettes, expression cassettes and  
10 plasmids. The transcriptional and translational initiation region (also sometimes referred to as a "promoter,"), preferably comprises a transcriptional initiation regulatory region and a translational initiation regulatory region of untranslated 5' sequences, "ribosome binding sites," responsible for  
15 binding mRNA to ribosomes and for translational initiation. It is preferred that all of the transcriptional and translational functional elements of the initiation control region are derived from or  
20 obtainable from the same gene. In some embodiments, the promoter will be modified by the addition of sequences, such as enhancers, or deletions of nonessential and/or undesired sequences. By "obtainable" is intended a promoter having a DNA sequence sufficiently similar to  
25 that of a native promoter to provide for the desired specificity of transcription of a DNA sequence of interest. It includes natural and synthetic sequences as well as sequences which may be a combination of synthetic and natural sequences.

30 For the transcriptional initiation region, or promoter element, any region may be used with the proviso that it provides the desired level of transcription of the DNA sequence of interest. The transcriptional initiation region may be native to or homologous to the  
35 host cell, and/or to the DNA sequence to be transcribed,

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or nonindigenous to the host cell or foreign or heterologous to the DNA sequence to be transcribed. By nonindigenous to the host cell is intended that the transcriptional initiation region is not normally found  
5 in the host into which the construct comprising the transcriptional initiation region is to be inserted. By foreign to the DNA sequence is intended a transcriptional initiation region that is not normally associated with the DNA sequence of interest. Efficient promoter  
10 elements for transcription initiation include the SV40 (simian virus 40) early promoter, the RSV (Rous sarcoma virus) promoter, the Adenovirus major late promoter, and the human CMV (cytomegalovirus) immediate early 1 promoter.

15 Inducible promoters also find use with the subject invention where it is desired to control the timing of transcription or the tissue or cell type in which the nucleic acid of interest is transcribed. Examples of inducible promoters include those obtainable from a  
20  $\beta$ -interferon gene, a heat shock gene, a metallothionein gene or those obtainable from steroid hormone-responsive genes, including insect genes such as that encoding the ecdysone receptor. Such inducible promoters can be used to regulate transcription of the transgene by the use of  
25 external stimuli such as interferon or glucocorticoids. Since the arrangement of eukaryotic promoter elements is highly flexible, combinations of constitutive and inducible elements also can be used. Tandem arrays of two or more inducible promoter elements may increase the  
30 level of induction above baseline levels of transcription which can be achieved when compared to the level of induction above baseline which can be achieved with a single inducible element.

Generally, the transcriptional regulatory sequence  
35 comprises DNA up to about 1.5 kb 5' of the

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transcriptional start of a gene, but can be significantly smaller. As necessary, this regulatory sequence may be modified at the position corresponding to the first codon of the desired protein by site-directed mutagenesis

5 (Kunkel, (1985) *Proc. Natl. Acad. Sci. (USA)*, 82:488-492) or by introduction of a convenient linker oligonucleotide by ligation, if a suitable restriction site is found near the N-terminal codon. In the ideal embodiment, a coding sequence with a compatible restriction site may be  
10 ligated at the position corresponding to codon #1 of the gene. This substitution may be inserted in such a way that it completely replaces the native coding sequence so that the substituted sequence is flanked at its 3' end by the gene terminator and polyadenylation signal.

15 Transcriptional enhancer elements optionally may be included in the transcription or expression cassette. By "transcriptional enhancer elements" is intended DNA sequences which are primary regulators of transcriptional activity and which can act to increase transcription from  
20 a promoter element, and generally do not have to be in the 5' orientation with respect to the promoter in order to enhance transcriptional activity. The combination of promoter and enhancer element(s) used in a particular cassette can be selected by one skilled in the art to  
25 maximize specific effects. Different enhancer elements can be used to produce a desired level of transgene transcription and/or expression in a wide variety of tissue and cell types. For example, the human CMV immediate early promoter-enhancer element can be used to  
30 produce high level transgene transcription and expression in many different tissues in vivo.

Examples of other enhancer elements which confer a high level of transcription on linked genes in a number of different cell types from many species include  
35 enhancers from SV40 and RSV-LTR. The SV40 and RSV-LTR

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are essentially constitutive. They can be combined with other enhancers which have specific effects, or the specific enhancers may be used alone. Thus, where specific control of transcription is desired, efficient

5 enhancer elements that are active only in a tissue-, developmental-, or cell-specific fashion include immunoglobulin, interleukin-2 (IL-2) and  $\beta$ -globin enhancers are of interest. Tissue-, developmental-, or cell-specific enhancers can be used to obtain transgene

10 transcription and/or expression in particular cell types, such as B-lymphocytes and T-lymphocytes, as well as myeloid, or erythroid progenitor cells. Alternatively, a tissue-specific promoter such as that derived from the human cystic fibrosis transmembrane conductance regulator

15 (CFTR) gene can be fused to a very active, heterologous enhancer element, such as the SV40 enhancer, in order to confer both a high level of transcription and tissue-specific transgene transcription. In addition, the use of tissue-specific promoters, such as LCK, allow

20 targeting of transgene transcription to T lymphocytes. Tissue specific transcription of the transgene can be important, particularly in cases where the results of transcription of the transgene in tissues other than the target tissue would be deleterious.

25 Tandem repeats of two or more enhancer elements or combinations of enhancer elements can significantly increase transgene expression when compared to the use of a single copy of an enhancer element; hence enhancer elements find use in the cassettes. The use of two

30 different enhancer elements, which can be either from the same or from different sources flanking or within a single promoter can in some cases produce transgene expression in each tissue in which each individual enhancer acting alone would have an effect, thereby

35 increasing the number of tissues in which transcription

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is obtained. In other cases, the presence of two different enhancer elements results in silencing of the enhancer effects. Evaluation of particular combinations of enhancer elements for a particular desired effect or  
5 tissue of expression is within the level of skill in the art.

Although generally it is not necessary to include an intron in the transcription and/or expression cassette, an intron optionally can be included which  
10 comprises a 5' splice site (donor site) and a 3' splice site (acceptor site) separated by a sufficient intervening sequence to produce high level extended in vivo transcription and/or expression of a transgene. Generally, an intervening sequence of about 100bp  
15 produces the desired expression pattern and/or level, but the size of the sequence can be varied as needed to achieve a desired result. The optional intron placed 5' to the coding sequence results in high level extended in vivo expression of a transgene but generally is not  
20 necessary to obtain expression. Optimally, the 5' intron specifically lacks cryptic splice sites which result in aberrantly spliced mRNA sequences.

If used, the intron splice donor and splice acceptor sites, arranged from 5' to 3' respectively, are  
25 placed between the transcription initiation site and the translational start codon. Alternatively, the intervening sequence can be placed 3' to the translational stop codon and the transcriptional terminator or inside the coding region. The intron can  
30 be a hybrid intron with an intervening sequence or an intron taken from a genomic coding sequence. An intron 3' to the coding region, particularly one of less than 100 bp, or any intron which contains cryptic splice sites can under certain condition substantially reduce the  
35 level of transgene expression produced in vivo. However,



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unexpectedly, a high level of *in vivo* expression of a transgene can be achieved using a vector that lacks an intron. Such vectors therefore are of particular interest for *in vivo* transfection.

5           Downstream from and under control of the transcriptional and/or translational initiation regulatory regions is a multiple cloning site for insertion of a nucleic acid sequence of interest which will provide for one or more alterations of host  
10   phenotype, for example to dramatically improve the treatment of acquired immune deficiency syndrome (AIDS), cystic fibrosis, cancer, heart disease, autoimmune diseases and a variety of life threatening infections. Conveniently, the multiple cloning site can be employed  
15   for a variety of nucleic acid sequences in an efficient manner. The nucleic acid sequence inserted in the cloning site can encode a ribozyme sequence or encode a polypeptide, for example, a protein having enzymatic activity, with the proviso that if the coding sequence  
20   encodes a polypeptide, it should lack cryptic splice sites which can block production of appropriate mRNA molecules and/or produce aberrantly spliced or abnormal mRNA molecules. The polypeptide can be one which is active intracellularly, a transmembrane protein, or it  
25   may be a secreted protein. It also can be a mutant protein, for example, which is normally secreted, but which has been altered to act intracellularly. The nucleic acid sequence can be DNA; it also can be a sequence complementary to a genomic sequence (an  
30   "antisense sequence"), where the genomic sequence may be one or more of an open reading frame, an intron, a non-coding leader sequence, or any other sequence where the complementary sequence will inhibit transcription, or messenger RNA processing, for example splicing, or  
35   translation.

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For the treatment of AIDS, anti-TAT, REV, TAR or other critical anti-HIV sequences may be used, particularly for expression of the appropriate coding sequences in T lymphocytes, macrophages and monocytes which can be achieved following iv administration of the appropriate coding sequences. Thus, DNA sequences which code for mRNA (an antisense or ribozyme sequence such as one to HIV-REV or a BCR-ABL sequence) or code for proteins such as transdominant negative mutants which specifically prevent the integration of HIV genes into the host cell genomic DNA, replication of HIV sequences, translation of HIV proteins, processing of HIV mRNA, or virus packaging in human cells can be used.

Expression of wild-type conductance regulator (CFTR) gene in the lungs of cystic fibrosis patients can be used in the treatment of cystic fibrosis (see Collins, (1992) *Science* 256:774-783). CFTR cDNA can be obtained from Dr. Collins at University of Michigan or Dr. Tsui at Toronto Sick Children's Hospital.

Expression of wild-type p53 in tumors of cancer patients with absent or aberrant expression of this gene can be used as a means of treating these patients. p53 is obtainable from Dr. Vogelstein at John Hopkins Univ. Other methods of cancer treatment include transcription of antisense sequences to over-expressed, transforming oncogenes, such as myc or ras in tumors.

Viral pneumonias remain a major cause of death and disability of the very young and in the aged population. Proteins such as granulocyte macrophage colony stimulating factor (GM-CSF), which stimulates production of white blood cells from the bone marrow of immunocompromised patients can be useful in controlling the course of viral infections. In particular, GM-CSF delivered via gene transfer would serve with prophylactic modalities for those at risk for occurrence of viral

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pneumonia, as well as in therapeutic modalities for treatment of the infection. GM-CSF also has significant anti-tumor activity.

Other examples of nucleic acid sequences of interest, include those encoding the LDL (low density lipoprotein) receptor, which specifically lowers serum cholesterol, and which can reduce the risk of heart attack in individuals with elevated serum cholesterol levels; sequences such as an antisense IL-1 receptor sequence to down-regulate the IL-1 receptor as a treatment of conditions involving inflammation, including sepsis, rheumatoid arthritis and asthma; downregulation of the gene coding for apo(a), circulating levels of which are correlated with the risk of coronary artery disease (CAD); and genes which block activity of activated T cell clones which attack myelin in multiple sclerosis or other targets in autoimmune diseases. A T-cell lymphocyte clone activated to recognize and attack myelin can be targeted by using an antisense sequence, ribozyme sequence or transgene coding for a transdominant negative mutant which specifically blocks surface expression on the T-cell of T-cell receptor components which mediate recognition and/or attack of myelin-sheathed cells. Other beneficial therapeutic nucleic acid sequences which can be expressed in appropriate cells in vivo using this invention include nucleic acid sequences encoding molecules which have superoxide dismutase activity or catalase activity to protect the lung from oxidant injury; endothelial prostaglandin synthase to produce prostacyclin and prostaglandin E<sub>2</sub>; and antiprotease alpha-1 antitrypsin, and erythropoietin.

The termination region which is employed primarily will be one of convenience, since termination regions appear to be relatively interchangeable. The termination region may be native to the intended nucleic

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acid sequence of interest, or may be derived from another source. Convenient termination regions are available and include the 3' end of a gene terminator and polyadenylation signal from the same gene from which the 5' regulatory region is obtained. Alternatively, a terminator and polyadenylation signal from a different gene or genes may be employed with similar results. Specific sequences which regulate post-transcriptional mRNA stability may optionally be included. For example, certain polyA sequences (Volloch and Housman, (1981) *Cell* 23:509-514) and  $\beta$ -globin mRNA elements can increase mRNA stability, whereas certain AU-rich sequences in mRNA can decrease mRNA stability (Shyu et al., (1989) *Genes and Devel.* 3:60-72). In addition, AU regions in 3' non-coding regions may be used to destabilize mRNA if a short half-life mRNA is desirable for a particular application.

The construct may additionally include sequences for selection, such as a neomycin resistance gene or a dihydrofolate reductase gene and/or signal sequences to generate recombinant proteins that are targeted to different cellular compartments or secreted when the wild type sequence is not. Any of a variety of signal sequences may be used which are well-known to those skilled in the art. These signal sequences may allow generation of new vaccine strategies or produce soluble antagonists directed against specific cell surface receptors such as transformed oncogenes. The sequences for selection may be on a separate plasmid and cotransfected with the plasmid carrying the therapeutic nucleic acid. Where a carrier is used, the selection plasmid may be complexed to a different carrier or to the same carrier as the therapeutic plasmid.

In some cases, it may be desirable to use constructs that produce long term transgene effects in vivo, either by integration of the transgene into host

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cell genomic DNA at high levels or by persistence of the transgene in the nucleus of cells in vivo in stable, episomal form. When desired, integration of the transgene into genomic DNA of host cells in vivo may be facilitated by administering the transgene in a linearized form (either the coding region alone, or the coding region together with 5' and 3' regulatory sequences, but without any plasmid sequences present). It may be possible to further increase the incidence of transgene integration into genomic DNA by incorporating a purified retroviral enzyme, such as the HIV-1 integrase enzyme, into the lipid carrier-DNA complex. Appropriate flanking sequences are placed at the 5' and 3' ends of the transgene DNA. These flanking sequences have been shown to mediate integration of the HIV-1 DNA into host cell genomic DNA in the presence of HIV-1 integrase. Alternatively, duration of transgene expression in vivo can be prolonged by the use of constructs that contain non-transforming sequences of a virus such as Epstein-Barr virus, and sequences such as oriP and EBNA-1, which appear to be sufficient to allow heterologous DNA to be replicated as a plasmid in mammalian cells (Burhans, et al., (1986) *Cell* 62:955-965).

The recombinant coding-sequence flanked at its 5' end by the promoter and regulatory sequences and at its 3' end by a terminator and regulatory sequences may be introduced into a suitable cloning plasmid (e.g., pUC18, pSP72) for use in direct DNA uptake in host cells following introduction into the host. It is a theory of the invention that the naked nucleic acid when introduced into the host is protected from degradation by nucleases by associating with lipid carriers in the blood, such as chylomicrons. Thus, more effective transfection may be achieved when lipid in the blood is at an elevated level after ingestion of food.

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The nucleic acid construct also may be complexed with a carrier such as a lipid carrier, particularly a cationic lipid carriers to form a complex. By a complex is intended an association between a nucleic acid construct such as a plasmid containing an expression cassette or a transcriptional cassette and a lipid mixture. The physical form of the complex may be a liposome with nucleic acid complexed to the outside or entrapped within the liposome, or the complex may be in the form of interleaved lipid and nucleic acid, or the complex may be a mixture of any or all of the above physical forms. For intravenous administration, generally the complex is prepared by sonicating the lipid mixture to be used and then mixing the sonicated mixture with the nucleic acid in an appropriate DNA:lipid ratio in a physiologically acceptable diluent immediately prior to use. The lipid carriers can be prepared from a variety of cationic lipids, including DOTAP, DOTMA, DDAB, L-PE, and the like. Lipid carrier mixtures containing a cationic lipid, such as N-[1-(2,3-dioleoyloxy) propyl]-N,N,N-triethylammonium chloride (DOTMA) also known as "lipofectin", dimethyl dioctadecyl ammonium bromide (DDAB), 1,2-dioleoyloxy-3-(trimethylammonio) propanol (DOTAP) or L-lysinyloxyphosphatidylethanolamine (L-PE) and a second lipid, such as dioleoylphosphatidylethanolamine (DOPE) or cholesterol (Chol), are of particular interest. DOTMA synthesis is described in Felgner, et al., (1987) *Proc. Nat. Acad. Sciences, (USA)* 84:7413-7417. DOTAP synthesis is described in Stamatatos, et al., *Biochemistry*, (1988) 27:3917-3925. DOTMA:DOPE lipid carriers can be purchased from, for example, BRL. DOTAP:DOPE lipid carriers can be purchased from Boehringer Mannheim. Cholesterol and DDAB are commercially available from Sigma Corporation. DOPE is commercially available from Avanti Polar Lipids.

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DDAB:DOPE can be purchased from Promega. Biodegradable cationic amphophiles also can be used (see, for example, copending PCT application attorney docket number MEBI-002/00WO filed December 17, 1992).

5 Lipid carriers such as cationic liposomes can mediate high level cellular expression of transgenes or mRNA by delivering the nucleic acid into a wide variety of cells in culture. The use of specific cationic lipids can confer specific advantages for *in vivo* delivery of  
10 complexes. For example, iv injection of nucleic acid complexed to DOTAP-containing liposomes or ethylphosphatidylcholine (E-PC) lipid carriers can target transgene expression primarily to the lung. Furthermore, DOTAP, as well as L-PE and cholesterol ester  $\beta$ -alanine  
15 (CEBA) are fully metabolized by cells, whereas DOTMA cannot be fully metabolized by cells. Therefore, DOTAP, E-PC, and L-PE, but not DOTMA, are suitable for repeated injection into mammalian hosts. Additionally, using a lipid carrier comprising a cationic lipid and a second  
20 lipid, particularly cholesterol or DOPE, can maximize transgene expression *in vivo*. Also, mixing a steroid, such as cholesterol, instead of DOPE, with DOTAP, DOTMA, or DDAB, substantially increases transgene expression *in vivo*.

25 Particular cells and tissues can be targeted, depending upon the route of administration and the site of administration. For example, a tissue which is closest to the site of injection in the direction of blood flow can be transfected in the absence of any  
30 specific targeting. Additionally, if desired, the lipid carriers may be modified to direct the complexes to particular types of cells using site-directing molecules. Thus antibodies or ligands for particular receptors or other cell surface proteins may be employed, with a  
35 target cell associated with a particular surface protein.

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For example, the AIDS virus is primarily directed to cells having the CD4 surface protein. By having anti-CD4 antibody bound to the surface of the lipid carrier, a nucleic acid lipid carrier complex can be directed  
5 primarily to T-helper cells.

A particular ligand or antibody can be conjugated to the lipid in accordance with conventional ways, either by conjugating the site-directing molecule to a lipid or by providing for a linking group on a lipid present in  
10 the bilayer for linking to a functionality of the site-directing compound. Such techniques are well known to those skilled in the art. Ligand-directed DNA-polycation complexes have been shown to transfect to hepatocytes in the liver after iv injection; the ability to transfect  
15 other cell types or tissue types by this approach has not been demonstrated.

Non-cationic lipid carriers, particularly pH sensitive liposomes, offer another potentially attractive approach to *in vivo* gene therapy. However, as compared  
20 to cationic liposomes, pH sensitive liposomes are less efficient in capturing DNA and delivering DNA intracellularly and may be inactivated in the presence of serum, thus limiting their *iv* use.

A number of factors can affect the amount of  
25 expression in transfected tissue and thus can be used to modify the level of expression to fit a particular purpose. Where a high level of expression is desired, all factors can be optimized, where less expression is desired, one or more parameters can be altered so that  
30 the desired level of expression is attained. For example, if high expression would exceed the therapeutic window, then less than optimum conditions can be used. The factors which can be modified are as follows:

Either the lipid composition of the complex or the  
35 mean diameter of the lipid carriers (when in particle



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form such as a liposome) injected can dramatically affect the level of transgene expression produced in vivo. Thus, the liposomal lipid compositions generally have a composition of 50% molar ratio of cationic lipid to non-cationic lipid, but may range from 5% to 100%. The diameter of the lipid carriers should generally be within the range of 100nm to 10  $\mu$ m. Cationic lipid carrier-DNA complexes wherein the lipid carriers range from 100 nm to several  $\mu$ m in diameter can produce significant levels of transgene expression after systemic introduction into a mammalian host.

The use of lipid carriers of greater than 500 nm (i.e. multilamellar vesicles (MLV) or large unilamellar vesicles (LUV)) can in certain cases significantly increase the level of transgene expression achieved in a mammalian host when compared to small unilamellar vesicles (SUV). MLV and LUV are prepared by vortexing rather than sonicating after addition of the aqueous material to the dry lipid film. Where it is desired to use particles, the resulting lipid carriers can be extruded under high pressure through sized polycarbonate membranes to achieve particular uniform size distributions for a particular use.

The use of particular nucleic acid to lipid carrier ratio also can increase the amount of transfection and/or the level of transcription and/or expression of a nucleic acid sequence of interest. The ratios used determine whether and to what level transgenes are expressed in vivo thus can be optimized, depending upon various factors including the nature of the construct, the size and lipid composition of the lipid carrier and whether it is MLV or SUV, the route of administration and the host mammal. As an example, using a reporter gene CAT (chloramphenicol acetyl transferase), an approximately 1:1 (range 0.5:1 to 2:1) DNA to lipid

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carrier ratio ( $\mu\text{g}$  DNA to nmoles of the cationic lipid) produces the highest levels of gene expression in a mouse in all organs after ip administration, and an approximately 1:4 ratio, (range 2:1 to 1:7) produces the highest levels of gene expression in all organs after iv administration. In addition to achieving a high level of transgene expression in a wide variety of tissues using optimal conditions, the majority of all cells present in the lung, spleen, lymph nodes and bone marrow are transfected *in vivo*, as well as the majority of all endothelial cells present in the heart.

The DNA:lipid carrier ratio also can affect at what level transgenes are expressed in mammalian hosts after systemic injection of the complexes. Several factors are important in order to optimize the DNA:lipid carrier ratio for a particular expression level desired. Thus, specific DNA:lipid carrier ratios are required for each type of cationic lipid used as well as for each different lipid carrier size used. To optimize for maximum expression, for each lipid carrier composition used, DNA is mixed together with the lipid carriers in multiple different ratios, ranging from 6:1 to 1:10 ( $\mu\text{g}$  DNA to nmole cationic lipid), in order first to determine which ratios result in aggregation of the DNA:lipid carrier complexes. Ratios which result in aggregation cannot be used *in vivo*. The ratios which do not result in aggregation are tested in animal models to determine which of the DNA:lipid carrier ratios confers the desired level of transgene expression *in vivo*. For example, the optimal DNA:lipid carrier ratios for SUV for DOTMA:DOPE, DDAB:DOPE, DOTAP:DOPE, DOTAP:Cholesterol, L-PE:CEBA, DDAB:Cholesterol and L-PE:DOPE are 1:4, 1:3, (very low activity at all ratios), 1:6, 1:1, 1:5, and 2:1, respectively. DNA:lipid carrier complexes are made in appropriate physiologic solutions. The DNA:lipid carrier

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complexes are mixed in physiologic solutions (approximately 290 milliosmoles) which do not themselves induce aggregation of the DNA:lipid carrier complexes. The solutions include 5% dextrose in water or normal saline. Cell surface receptors for cationic lipid carriers can be used to both regulate and confer target cell specificity on transgene expression in mammalian hosts. Cationic lipid carrier:DNA complexes are internalized by cells by a classical receptor-mediated endocytosis (see Figure 7) using cell surface receptors which contain specific binding sites for, and are able to internalize, cationic molecules. Using agents such as cytokines, growth factors, other soluble proteins and certain drugs, it is thus possible to selectively up or down regulate these cation-binding receptors. The rate of up or down regulation of these receptors by the appropriate agent will allow selection of specific cells for enhanced or reduced levels of transfection *in vivo*. Cell surface receptors for naked DNA also can be used both to regulate and to confer target cell specificity on transgenic expression in mammalian host.

The most frequent interaction between DOTMA lipid carriers, either uni- or multilamellar lipid carriers, complexed to plasmid DNA and the various cell types (for example, CV-1 monkey kidney cells, U937 human myelomonocytic leukemia cells, K562, MEL (murine erythroblastic leukemia cells), rat alveolar macrophages, and alveolar type II cells), is that of lipid carrier adhesion and internalization. This interaction is common to well-defined examples of receptor-mediated endocytosis. All cells which appear to have contacted cationic lipid carrier:DNA complexes ingest the complexes following binding of the complexes to the plasma membrane. All these cell types demonstrate the same

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classical receptor-mediated endocytic pathway of internalization.

The mammalian host may be any mammal, particularly a mammal having symptoms of a genetically-based disorder  
5 or an infectious disease which is amenable to gene-based therapy. Thus, the subject application finds use in domestic animals, feed stock, such as bovine, ovine, and porcine, as well as primates, particularly humans. The mammalian host may be pregnant, and the intended  
10 recipient of the gene-based therapy may be either the gravid female or the fetus or both.

In the method of the invention, transfection in vivo is obtained by introducing a therapeutic transcription or expression vector into the mammalian  
15 host, either as naked DNA or complexed to lipid carriers, particularly cationic lipid carriers. The constructs may provide for integration into the host cell genome for stable maintenance of the transgene or for episomal expression of the transgene. The introduction into the  
20 mammalian host may be by any of several routes, including intravenous or intraperitoneal injection, intratracheally, intrathecally, parenterally, intraarticularly, intranasally, intramuscularly, topical, transdermal, application to any mucous membrane surface,  
25 corneal instillation, etc. Of particular interest is the introduction of a therapeutic expression vector into a circulating bodily fluid or into a body orifice or cavity, such as lung, colon, vagina, and the like. Thus, iv administration and intrathecal administration are of  
30 particular interest since the vector may be widely disseminated following such routes of administration, and aerosol administration finds use with introduction into a body orifice or cavity. Any physiologically acceptable medium may be employed for administering the DNA or lipid  
35 carriers, such as deionized water, saline, phosphate-

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buffered saline, 5% dextrose in water, and the like, depending upon the route of administration. Other components may be included in the formulation such as buffers, stabilizers, biocides, etc. These components  
5 have found extensive exemplification in the literature and need not be described in particular here. Any diluent or components of diluents that would cause aggregation of the complexes should be avoided, including high salt, chelating agents, and the like.

10 The amount of naked DNA or complexes used will be an amount sufficient to provide for adequate dissemination to a variety of tissues after entry of the DNA or complexes into the bloodstream and to provide for a therapeutic level of expression in transfected tissues.  
15 A therapeutic level of expression is a sufficient amount of expression to, prevent, treat or palliate a disease or infection of the host mammal. In addition, the dose of the nucleic acid vector used must be sufficient to produce a desired level of transgene expression in the  
20 tissue or tissues of interest, in vivo for example,  $\geq 1\text{mg}$  of an expression plasmid alone injected into a mouse results in a high level of expression of the CAT gene in multiple tissues. Other DNA sequences, such as adenovirus VA genes can be included in the administration  
25 medium and be co-transfected with the gene of interest. The presence of genes coding for the adenovirus VA gene product may significantly enhance the translation of mRNA transcribed from the plasmid if this is desired.

The level and tissues of expression of the  
30 recombinant gene may be determined at the mRNA level and/or at the level of polypeptide or protein. Gene product may be quantitated by measuring its biological activity in tissues. For example, enzymatic activity can be measured by biological assay or by identifying the  
35 gene product in transfected cells by immunostaining

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techniques such as probing with an antibody which specifically recognizes the gene product or a reporter gene product present in the expression cassette.

Alternatively, potential therapeutic effects of the gene product can be measured, for example where the DNA sequence of interest encodes GM-CSF, by determining the effects of gene expression on survival of lethally irradiated animals in which the GM-CSF transgene is expressed. Production of significant amounts of a transgene product will substantially prolong the survival of these mice.

Where expression of the polypeptide/protein or even the mRNA itself confers a changed biochemical phenotype upon the host, the presence of a new phenotype or absence of an old phenotype may be evaluated; for example, as a result of transfection of the host cells, there may be enhanced production of pre-existing desirable products formerly produced in insufficient quantities or there may be reduction or even suppression of an undesirable gene product using antisense, ribozyme or co-suppression technologies; in the case of suppression, a reduction of the gene product may be determined. Typically, the therapeutic cassette is not integrated into the host cell genome. If necessary, the treatment can be repeated on an *ad hoc* basis depending upon the results achieved. If the treatment is repeated, the mammalian host can be monitored to ensure that there is no adverse immune or other response to the treatment.

As an example, in a clinical setting where it is desired to treat a particular disease state, both the biological efficacy of the treatment modality as well as the clinical efficacy need to be evaluated, if possible. For example, in the treatment of cystic fibrosis, there is a generalized epithelial dysfunction which manifests itself as abnormalities in the electrolyte and water

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content of luminal liquid or "secretions" of the airways, sweat glands, intestinal and reproductive tracts, and pancreas. The biological efficacy of gene therapy therefore can be evaluated by for example measuring the

5 transepithelial electrical potential difference prior to treatment and following transaction. The evaluations can be done following transfection of nasal cells and also following transfection lung cells. Examples of techniques which can be used for measuring the

10 bioelectric potential difference across respiratory epithelial and cystic fibrosis as described in Knowles, et al., (1981) *New England General of Medicine* 305:1489-1495; Knowles, et al., (1983) *General of Clinical Investigation* 71:1410-1417; and Knowles, et al., (1983)

15 *Science* 221:1067-1070. The clinical efficacy, whether treatment of the underlying defect is effective in changing the course of disease, can be more difficult to measure. While the evaluation of the biological efficacy goes a long way as a surrogate end point for the clinical

20 efficacy, and is not definitive. Thus, measuring a clinical endpoint such as the so-called "spirometry" factors which give an indication of lung function of a for example, a six-month period of time, may give an indication of a clinical efficacy of the treatment

25 regimen. Typical measurements would include forced vital capacity (FVC) of the lung and forced expired volume in one second (FEV). An example of the type of clinical study which can be performed to evaluate the efficacy of gene therapy for cystic fibrosis is one which is being

30 used for amiloride for the treatment of lung disease and cystic fibrosis: Knowles, et al., (1990) *New England General of Medicine* 322:1189-1194. Similarly, one skilled in the art can evaluate the biological and clinical efficacy of a particular gene therapy protocol.

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The subject compositions can be provided for use in one or more procedures. Kits will usually include the DNA either as naked DNA or complexed to lipid carriers. Additionally, lipid carriers may be provided in a  
5 separate container for complexing with the provided DNA. The DNA either for direct administration or for complexing with lipid carriers, or the lipid carrier/DNA complexes may be present as concentrates which may be further diluted prior to use or they may be provided at  
10 the concentration of use, where the vials may include one or more dosages. Conveniently, single dosages may be provided in sterile vials so that the physician or veterinarian may employ the vials directly, where the vials will have the desired amount and concentration of  
15 agents. Thus, a vial may contain the DNA or the DNA/lipid carrier complexes in appropriate proportional amounts. When the vials contain the formulation for direct use, usually there will be no need for other reagents for use with the method.

20 The invention finds use in *in vivo* prevention, treatment and/or palliation of a number of diseases. *In vivo* replacement of a gene can be accomplished by techniques such as homologous recombination or initial knockout of the aberrant gene and subsequent replacement  
25 with the desired transgene. An additional benefit of expressing nucleic acids in appropriate cells *in vivo* using this invention, is that the encoded proteins would be processed and subject to post translational modification in the correct manner.



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The following examples are offered by way of illustration and not by limitation.

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Example 1Preparation of Plasmids for in vivo Gene Therapy

Details regarding the plasmids that have been used for transfection of mammalian cells are as follows.

- 5        pRSVCAT: construction of this plasmid is described in Gorman et al., (1982) *Proc. Nat. Acad. Sciences (USA)* 79:6777-6781. In the pRSVCAT plasmid, the 3'-RSVLTR is juxtaposed as a promoter upstream from CAT encoding sequences. The distance between the LTR transcriptional  
10 start site and the CAT initiation codon (the first AUG downstream from the start site) is about 70 bp.

p5'PRL3-CAT: construction of this plasmid is described in Sakai et al., (1988) *Genes and Development* 2:1144-1154.

- 15        pSIS-CAT: construction of this plasmid is described in Huang and Gorman, *Nucleic Acids Research*, (1990) 18:937-948.

- pZN20: construction of this plasmid is illustrated in Figure 5. The plasmid was prepared as follows.  
20 pCATwt760 (Stinski and Roehr, (1985) *J. Virol.* 55:431-441) was treated with *Hind*III and the fragment containing the HCMV IE 1 enhancer and promoter element purified. The isolated fragment was then cloned into the *Hind*III site of pSP72 (Promega) creating pZN9. Clones were  
25 screened in which the enhancer and promoter element is as shown in Figure 5. Following partial *Hind*III digestion of pZN9, the blunt ends were filled in with DNA polymerase I Klenow fragment. The resulting clone pZN12 had lost the *Hind*III site 5' to the enhancer and promoter  
30 element. pZN12 was then treated with *Nco*I and *Hind*III and the large *Nco*I-*Hind*III fragment purified and ligated to a purified small *Nco*I-*Hind*III fragment from pBC12/CMV/IL-2 (Cullen, (1986) *Cell* 46:973-982. pBC12/CMV/IL-2 contains the HCMV promoter from the AD169

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strain. The resulting clone was pZN13. pZN13 was partially digested with BamHI, filled in with DNA polymerase I Klenow fragment and the resulting clones screened for the clone which had lost the BamHI site at the 5' end of the enhancer and promoter element. The resulting clone was called pZN17. pZN17 was treated with HindIII and BamHI and the resulting HindIII-BamHI large fragment was purified and ligated to a purified small HindIII-BamHI fragment obtained from pSV2-CAT (Gorman, et al., (1982), *Molecular Cell Biology* 2:1044-1051). The resulting clone was pZN20. The full restriction map of HCMV (Towne) is shown in Figure 6A. HCMV (AD169) is shown in Figure 6C. A comparison of the two promoters is shown in Figure 6B. Significantly more expression is obtained when a promoter from the AD169 strain is used as compared to one from the Towne strain. pZN20 contains a composite promoter which has the Towne sequence 5' of the NcoI site and the AD169 sequence 3' of the NcoI site. The NcoI site is indicated by the asterisk in Figure 6B. pZN20 has this composite HCMV promoter followed by the CAT gene, SV40 t-intron and SV40 polyA adenylation site.

pZN27: Construction of this plasmid is illustrated in Figure 9. pZN27 contains the composite HCMV promoter followed in order by the SV40 t-intron, the CAT coding sequence and the SV40 polyA adenylation site.

pZN46: Construction of this plasmid is shown in Figure 10A and Figure 10B. pZN46 contains the composite HCMV promoter, followed by the human IL-2 gene, rat preproinsulin 2 intron and polyA addition site from the rat preproinsulin 2 gene. These last three components were derived from the pBC12/CMV/IL-2 plasmid of Cullen (Cell, (1986) 46:973-982. The rat preproinsulin 2 intron was modified by deleting an internal 162 base pair NdeI fragment.

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pZN32: Construction of this plasmid is shown in Figure 11. pZN32 contains the composite HCMV promoter followed in order by the modified rat preproinsulin 2 intron described for pZN46, human CFTR cDNA, and rat preproinsulin 2 gene polyA addition site as described for pZN46. CFTR cDNA was obtained from pBQ4.7 from F. Collins (Univ. of Michigan).

pZN51: Construction of this plasmid is shown in Figure 13. pZN51 contains the composite HCMV promoter followed by the CAT coding sequence and the SV40 polyA adenylation site.

pZN60, pZN61, pZN62, pZN63: Construction of these plasmids is shown in Figure 14. pZN60 contains the HCMV composite promoter followed by the modified rat preproinsulin 2 intron, the CAT coding sequence, and the SV40 polyA addition site. pZN61 is identical to pZN60 but contains an additional 166 base pairs 5' to the intron. This additional DNA is the 166 base pairs immediately 5' of the intron in the pBC12/CMV/IL-2 plasmid and contains rat preproinsulin 2 gene coding sequence. pZN62 is similar to pZN60 except that the intron is 3' of the CAT coding sequence rather than 5' as in pZN60. pZN63 is identical to pZN62 except for the additional 166 base pairs 5' to the intron. This is the same additional sequence described for pZN61.

pCIS-CAT: This plasmid was made as described in Huang, M.T.F. and Gorman, C.M., (1990) *Nucl. Acids Res.* 18:937-947, with the exception that a CMV promoter and a hybrid intron sequence were used rather than the SV40 promoter in the plasmid pML.I.CAT, Gorman, et al., (1990) *DNA Protein Eng. Tech.*, 2:3-10.

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Example 2Preparation of Lipid carriers and DNA Complexing with Lipid carriers

Lipid carriers containing a cationic lipid, such  
5 as N-[(1-2-3-dioleyloxy) propyl]  
-N,N,N-triethylammonium chloride (DOTMA), dimethyl  
dioctadecyl ammonium bromide (DDAB), or  
1,2-dioleoyloxy-3-(trimethylammonio)propane (DOTAP) or L-  
lysinyphosphatidylethanolamine (L-PE) and a second  
10 lipid, such as dioleoylphosphatidylethanolamine (DOPE) or  
cholesterol, were prepared as follows.

Preparation of lipid carriers:

Lipids, e.g. DDAB, L-PE, cholesterol-ester- $\beta$ -  
alanine (CEBA), DOTAP, and cholesterol (Chol) were  
15 dissolved in chloroform. Proper amounts of each lipid  
(determined by the desired molar ratio of each lipid in  
the final lipid carrier formulation usually 1 to 1 moles  
cationic lipid to moles non-cationic lipid but ranging  
from 5 to 1 up to 1 to 5) were mixed together and  
20 evaporated to dryness on a rotary evaporator. The lipid  
film was then resuspended by vortexing after the addition  
of 5% dextrose in water or lipid carrier buffer (25 mM  
Tris-HCl pH 7.4, 100  $\mu$ M ZnCl<sub>2</sub>, NaCl to isotonicity. To  
make a final lipid concentration of 20mM of multi-  
25 lamellar vesicles (MLV). For the preparation of small  
unilamellar vesicles (SUV), the mixture was then  
sonicated in a bath sonicator for 15 min, and the lipid  
carriers were stored under argon at 4°C until use.

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Plasmid Preparation:

The *E. coli* strain HB101 which carried the plasmid was grown in TB at 37°C. The method of plasmid purification is a modification of the protocol of "lysis by alkali" and "purification of plasmid DNA by precipitation with polyethylene glycol" described by Sambrook et al., (1989) (*Molecular Cloning*, 2nd edition, Cold Spring Harbor Laboratory Press). The modification was that the precipitation of DNA by PEG was omitted.

10 The final DNA preparation was dissolved in 10mM Tris-HCl pH 8.0.

Preparation of lipid carrier-plasmid complexes:

Plasmids were diluted separately in 5% dextrose in water solution to the desired concentration (usually 1 µg/µl). The lipid carriers were also diluted in 5% dextrose in water to the same volume as the plasmid.

The amounts of lipid carriers used were determined based on the ratio of nmoles of lipid to µg of plasmid added, e.g. for lipid carrier:plasmid=1:1, one nmole of cationic lipid is mixed with 1 µg of plasmid DNA.

20 Plasmid and lipid carriers were then mixed together to form DNA:lipid carrier complexes.

Dose injected. At least 50 µg, and routinely 100 µg of plasmid DNA complexed to cationic lipid carriers were injected intravenously by tail vein per mouse. For injection of plasmid alone, at least 500 µg and routinely 2 mg of plasmid DNA were injected intravenously by tail vein per mouse.



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Example 3Demonstration by Immunohistochemistry of CAT Gene  
Expression in theLung After Intravenous (iv) injection of pZN27-DDAB:5                   Cholesterol Lipid carrier Complexes

Lipid carrier: DDAB:Cholesterol=1:1, stock 20 mM in lipid carrier buffer.

Plasmid: pZN27.

DNA:Lipid carrier Ratio: lipid carrier:plasmid=5

10 nanomoles cationic lipid:1 µg DNA

DNA dose: 100 µg plasmid DNA in 200 µl 5% dextrose in water was injected iv by tail vein per mouse.

Mice: ICR, female, 25 grams.

15 Immunohistochemical staining to detect CAT protein in  
lung sections of mice treated in vivo.

Procedure: Forty eight hours after injection of the pZN27-DDAB:Cholesterol complexes, the lungs are removed, perfused with 33% O.C.T. Embedding Medium (Miles, Inc.), embedded in O.C.T. and snap frozen. Frozen tissues were  
20 sectioned at 6 µm, collected onto glass slides, fixed for 10 minutes in 4°C acetone and then placed in 0.2% Triton X-100 to permeabilize membranes. Sections were then incubated for 12-48 hours with the monoclonal anti-CAT antibody (available from Dr. Parker Antin, Univ. of  
25 Arizona) or isotype negative control antibody at the appropriate dilution. After washing, a biotinylated antibody directed against the primary antibody (Zymed, S. San Francisco) was added for a minimum of 60 minutes, followed by application of the streptavidin-alkaline  
30 phosphatase complex (Zymed) for 60 minutes. The substrate-chromogen appropriate for the enzyme label was then applied, per the manufacturer's instructions.

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Slides were coverslipped in water-soluble mounting media for examination.

Results: The results are shown in Figure 1 and demonstrate diffuse staining of the lung. The stain  
5 localizes to the alveolar walls, indicating that greater than 70% of pulmonary vascular endothelial cells, as well as alveolar lining cells, including type I and type II cells and alveolar macrophages are transfected by a single iv injection of DNA lipid carrier complexes. In  
10 addition, significant numbers of bronchiolar airway lining cells stain positively for CAT protein, and are therefore transfected in vivo by iv injection of lipid carrier:DNA complexes. Thus, the great majority of all cells in the lung transfected by an iv injection of  
15 pZN27-DDAB:Cholesterol complexes.

#### Example 4

##### Expression of pCIS-CAT

##### Following Intraperitoneal Administration

Effect of the Amount of pCIS-CAT-cationic lipid carrier  
20 complexes injected ip on the level of CAT gene expression in vivo.

Female ICR mice were injected ip with 1 ml of 5% dextrose in water containing 0.01, 0.1, or 1 mg of pCIS-CAT expression plasmid complexed with 0.01, 0.1 or 1  
25  $\mu$ moles, respectively, of DDAB:DOPE lipid carriers. Mice were sacrificed 48 hours later, the organs removed, and tissues were homogenized in 0.25M Tris-HCl buffer pH 7.8, using a hand-held homogenizer. Cytoplasmic extracts were made, normalized by protein content and the level of CAT  
30 activity then measured. The experiments comprise three animals per group and the results show the mean dpm  $\pm$  SEM of acetylated chloramphenicol.

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**Methods:** Lipid carriers containing DDAB were prepared in 1:1 molar ratio with DOPE, as follows: 10  $\mu$ moles of DOPE dissolved in chloroform and 10  $\mu$ moles of the cationic lipid, dissolved in ethanol were evaporated to dryness on a rotary evaporator. One ml sterile of water was added, and the mixture was sonicated in a bath sonicator (Laboratory Supply, Hicksville, NY) for 20 min. Lipid carriers had mean diameters of approximately 100  $\pm$  25 nm. For CAT assay, cell extracts were made, and their protein content determined by the Coomassie Blue assay (BioRad, Richmond). One hundred  $\mu$ g of protein from the lung, spleen, liver, and heart extracts, and 50  $\mu$ g of lymph node extract were reacted with  $^{14}$ C labeled chloramphenicol and chromatographed as previously described (Gorman, supra). To calculate dpm, both the acetylated and unacetylated species were cut from TLC plates and radioactivity counted in a scintillation counter.

**Results:** To assess potential dose-response relationships *in vivo*, animals were injected animals in groups of three with 0.01 mg, 0.1 mg, or 1 mg of pCIS-CAT plasmid complexed with 0.01  $\mu$ mole, 0.1  $\mu$ mole, or 1  $\mu$ mole respectively of DDAB:DOPE lipid carriers. Both the 0.1 mg and 1 mg DNA doses produced highly significant levels of CAT protein ( $p < 0.005$ ) in all the organs assayed. Maximal levels of CAT gene expression in each organ were produced by the 1 mg DNA dose: increasing the DNA-lipid carrier dose 10 fold resulted in an approximately 2 fold increase in lymph node CAT levels and a 3 fold increase in the spleen.

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Example 5Demonstration of CAT gene expression in the spleen after intravenous (iv) injection of p5'PRL3-CAT:L-PE:CEBA complexes.

- 5 Lipid carrier: L-PE:CEBA=1:1, stock 20 mM in lipid carrier buffer.
- Plasmid: p5'PRL3-CAT. (Sakai, et al., (1988) Genes and Development 2:1144-1154)
- DNA:Lipid carrier Ratio: lipid carrier:plasmid=1 nmole
- 10 cationic lipid:  
1 µg plasmid DNA.  
DNA dose: 200 µg plasmid DNA in 200 µl 5% dextrose in water was injected by tail vein per mouse.  
Mice: Balb/c, female, 25 grams.
- 15 Tissue extraction procedure: Forty eight hours after tail vein injection, mice were sacrificed, whole spleens were homogenized in 1ml of 0.25M Tris-HCl pH 7.8, 5mM EDTA, 80 µg/ml PMSF and the resulting extract was centrifuged and then the supernatant was subjected to 3
- 20 cycles of freeze-thaw and then heated to 65°C for 20 min.  
CAT assay procedure: 100 µl of extract+10 µl of 20 mM acetyl CoA+4 µl of <sup>14</sup>C-chloramphenicol (25 µCi/ml, 55 mCi/mole, Amersham) were incubated together at 37°C for 6 hr. At 3 hours, an additional 10 µl of acetyl CoA
- 25 was added.
- Results:  
This experiment showed that a significant level of CAT activity was present in the spleen extract of the treated animal, but not in the extract of control spleen,
- 30 taken from an animal injected with lipid carrier alone.

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Example 6Injection of DOTMA:DOPE+pSIS-CAT Plasmid Clearly Did Not  
ProduceDetectable CAT Gene Expression in vivo)

- 5 Lipid carrier: DOTMA:DOPE=1:1, in 5% dextrose in water  
Plasmid: pSIS-CAT (Huang, and Gorman, (1990) *Nucleic  
Acids Research* 18:937-947).  
Ratio: Cationic lipid:plasmid=4 nmoles: 1  $\mu$ g, dose:  
100  $\mu$ g DNA in 200  $\mu$ l 5% dextrose in water.
- 10 Mouse: ICR, female, 25 grams.  
Injection: tail vein.  
Tissue collection and processing: Mice were sacrificed at  
day 2 and day 6, and lung, spleen, liver, and heart were  
collected. The whole organs were homogenized in 0.5 ml,  
15 except livers which were homogenized in 2.0 ml, of 0.25M  
Tris-HCl pH 7.8, 5 mM EDTA, 2  $\mu$ g/ml aprotinin, 1  $\mu$ g/ml E-  
64, and 0.5  $\mu$ g/ml leupeptin (all protease inhibitors  
purchased from Boehringer Mannheim). Extracts were  
subjected to three cycles of freeze-thaw, then heated to  
20 65°C for 10 min.  
CAT assay: 100  $\mu$ l of extracts for each assay with 0.3  
 $\mu$ Ci of  $^{14}$ C-chloramphenicol and 10  $\mu$ l of 20 mM acetyl CoA  
at 37°C for either 5 hrs or 24.5 hrs, and the materials  
were then extracted using ethyl acetate and analyzed on  
25 TLC plates.

Results:

- There were no acetylated chloramphenicol species  
presented as determined by comparing the extracts from  
treated animals with that from control animals. Thus,  
30 under similar experimental conditions that produce high  
level expression of pZN27, the use of the pSIS-CAT  
expression vector does not result in any detectable  
expression of the linked-CAT gene in any of the tissues  
assayed in vivo. The lack of expression of pSIS-CAT in

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vivo may be due either to a different promoter-enhancer element (SV40) or to a different intron sequence when compared to the pZN27 vector, which yields high level in vivo expression.

5

Example 7Interaction Of DNA: Lipid carrier Complexes  
With Cell Surface Receptors

Cells and cell culture: CV-1 (African green monkey kidney), U937 (human myelocytic leukemia), murine erythroleukemia (MEL) cells, and K562 cells (human erythroleukemia cells were obtained from the American Type Culture Collection (Rockville, MD). CV-1 and MEL cells were maintained in Dulbecco minimum essential medium DME-H-21 with 5% fetal bovine serum (FBS) at 37°C and 7% CO<sub>2</sub>. Rat alveolar type II cells and rat alveolar macrophages were isolated and purified as previously described. (Debs et al., (1987) *Amer. Rev. Respiratory Disease* 135:731-737; Dobbs, (1986) *Amer. Rev. Respiratory Disease* 134:141-145) Type II cells were maintained in DME-H-16 with 5% FBS at 37°C and 7% CO<sub>2</sub>. Twenty nmole of DOTMA:DOPE lipid carriers complexed to 20 µg of pRSV-CAT plasmid DNA were added to 2 million cells growing in 60 mm Falcon plastic dishes (either SUV or MLV), and fixed for EM at time points from 15 minutes to 2 hours thereafter.

25

Fixation and Processing for Electron Microscopy

DOTMA lipid carriers and cells in tissue culture or freshly isolated from blood or pulmonary alveoli were fixed in 1.5% glutaraldehyde in 0.1 molar sodium cacodylate buffer containing 1% sucrose, pH 7.4, at room temperature for 1 hr. Following tannic acid and uranyl acetate enhancement, tissue was dehydrated in a graded series of alcohols and embedded in epoxy 812 resin

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(Ernest F. Fullam, Inc., Latham, NY) sectioned on an MT 2 microtome using diamond knives and examined with a Jeol 100CX transmission electron microscope operating at 80 kV. Electron photomicrographs of the internalization of lipid complexes in CV-1 monkey kidney cells are shown in Figure 7. The arrows in panel (a) show particles binding to clathrin-coated pits; in panel (b), the arrows show where the particles have been ingested and are present in endosomes.

10       The most frequent interaction between DOTMA lipid carriers, either uni- or multilamellar lipid carriers, complexed to plasmid DNA and the various cell types (CV-1 monkey kidney cells, U937 human myelomonocytic leukemia cells, K562, MEL erythroblastic leukemia cells, rat  
15   alveolar macrophages, and alveolar type II cells), is that of lipid carrier adhesion and internalization in a typical coated vesicle pathway (Fig. 7 a-f). This interaction is common to well defined examples of receptor-mediated endocytosis. All cells which appear to  
20   have contacted cationic lipid carrier:DNA complexes ingest the complexes after binding to the plasma membrane. All these cell types (derived from rodent, monkey and human cells, demonstrated the same classical receptor-mediated endocytic pathway of internalization.  
25   DNA-cationic liposome complexes were generally taken up by human cells as well as or better than nonhuman and particularly rodent cells of similar lineage.

#### Example 8

Demonstration that Mouse T Lymphocytes are Transfected in  
30 vivo.

Female ICR mice were injected ip with 1 ml of 5% dextrose in water containing 1 mg of a pZN27 plasmid complexed to 1  $\mu$ mole of DDAB:DOPE (1:1 molar, SUV) lipid

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carriers. Mice were sacrificed 48 hrs. later, the spleen and lymph nodes were removed, and rendered into single cell suspensions by homogenizing in serum containing medium. The cells were then incubated with FITC-  
5 conjugated anti-Thy 1.2 antibody (provided by Dr. J. Beck, San Francisco Veterans Administration Medical Center) and sorted by FACS. The Thy 1.2<sup>+</sup> T lymphocyte fraction was cytopun onto microscope slides and fixed and probed for the presence of intracellular CAT protein  
10 after permeabilizing the cells using 0.25% Triton X-100. The cells were incubated with an anti-CAT monoclonal antibody (gift from Dr. P. Antin, Univ. of Arizona) for 1 hr. at 20°C and then stained with a Texas Red- conjugated goat-anti-mouse IgG for 1 hr. at 20°C. Figure 2A shows a  
15 field of T lymphocytes by phase contrast microscopy and 2B shows the same field viewed by fluorescence microscopy. These results demonstrate that more than 70% of Thy 1.2<sup>+</sup> T lymphocytes are transfected in vivo (as shown by the red fluorescence), by a single ip injection  
20 of pZN27:DDAB:DOPE complexes. Thy 1.2<sup>+</sup> lymphocytes from untreated mice do not show immunofluorescent staining as shown in 2D; a phase contrast micrograph of the same field is shown in 2C.

#### Example 9

#### 25 Demonstration that Mouse Hematopoietic Bone Marrow-Derived

#### Cells Are Transfected in vivo.

Female ICR mice were injected ip with 1 ml of 5% dextrose in water containing 1 mg of a pZN27 plasmid  
30 complexed to 1  $\mu$ mole of DDAB:DOPE SUV lipid carriers. Mice were sacrificed 48 hrs. later, bone marrow-derived hematopoietic cells were then obtained by perfusing the femur cavity with RPMI-1640 medium and then homogenizing



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clumps to obtain a single cell suspension. Bone marrow cells were then centrifuged onto glass slides and fluorescently stained as described in Example 8. This experiment demonstrated that approximately 20% of mouse  
5 bone marrow hematopoietic cells (including cells that on the basis of morphology are primitive myeloblastic and erythroblastic precursor cells) were transfected *in vivo* by a single ip dose of pZN27:DDAB:DOPE complexes.

#### Example 10

- 10 Demonstration that Human CD4<sup>+</sup> T Lymphocytes, Freshly Isolated  
from Normal Donors, are Transfected *in vitro*.

Buffy coat preparations were freshly isolated from normal human donors by gradient centrifugation. The  
15 cells were then panned using an anti-CD3 (Becton-Dickinson, Mountain View, CA) monoclonal antibody to isolate the CD3<sup>+</sup> T lymphocyte fraction. These cells were then transfected using the following protocol: 10 million cells were plated on 100 mm dishes and then 25 µg of  
20 pZN27 complexed to 50 nmoles of DDAB:DOPE (1:1) SUV lipid carriers were added for 48 hours. Control cells were not transfected. The cells were then incubated with a FITC-conjugated monoclonal anti-CD4 antibody (Becton-Dickinson) and sorted by FACS. The resulting CD4<sup>+</sup> T  
25 lymphocytes were cytospun onto microscope slides and fixed and probed for the presence of intracellular CAT protein after permeabilizing the cells using 0.25% Triton x-100. The cells were incubated with an anti-CAT monoclonal antibody for 1 hr at 20°C and then stained  
30 with a Texas Red conjugated goat anti-mouse IgG for 1 hr at 20°C.

#### Results:

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Results are shown in Figure 4 and demonstrate that at least 70% of freshly isolated human CD4<sup>+</sup> T lymphocytes are transfected after exposure to pZN27:DDAB:DOPE complexes in culture as shown by the red fluorescence of 5 cells in 4B. Control untransfected cells showed no fluorescence. These results suggest that this approach may dramatically improve the therapy of diseases including AIDS and cancer.

As the above results show, high level transgene 10 expression has been achieved in heart, kidney, lymph nodes, bone marrow cells, liver, lung and spleen after systemic (iv or ip) transgene administration. Transfection of the heart, kidney, lymph nodes or bone marrow cells individually after systemic (iv or ip) 15 transgene administration into adults has not previously been accomplished. Transfection of T lymphocytes, lung airway or alveolar cell types, cardiac endothelial lining cells and cardiac muscle cells, and bone marrow hematopoietic precursor cells *in vivo* by systemic 20 administration of DNA has not been shown previously. Specifically, greater than 50% of T lymphocytes, lung airway epithelial, alveolar and vascular endothelial cell types, cardiac endothelial lining cells and bone marrow hematopoietic precursor cells (including about 70% of 25 blast cells) are transfected *in vivo*, following one iv or ip injection of CAT expression plasmid-cationic lipid carrier complexes. Transfection of a high percentage of all the cells present in any single tissue has not been reported previously.

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Example 11

Efficient transfection of a variety of human lung cancer cell lines using cationic liposome-mediated delivery of DNA.

5 Method:

Cell Culture: NCI-H69, NCI-H82, and NCI-H520 cells were used. H69 and H520 cells were grown in RPMI-1640 with 10% fetal bovine serum (FBS) and H82 cells were grown in Dulbecco's minimum essential medium (DME)-H21 with 10%

10 FBS.

Liposome preparation: Liposomes were prepared as follows: a total of 4  $\mu$ moles of lipid dissolved in chloroform, (or in ethanol (DOTMA)) were evaporated to dryness on a rotary evaporator. One ml of 50 mM Tris, 15 0.5 mM EDTA, 50 mM NaCl, 100  $\mu$ M ZnCl<sub>2</sub> buffer per 20  $\mu$ moles of lipid was added, and the mixture was sonicated in a bath sonicator (Laboratory Supply Co., Hicksville, NY) for 20 min. The resulting liposomes have an approximate mean diameter of 100  $\pm$  25 nm. The following 20 liposome preparations were used: pure DOTMA, DOTMA:Cholesterol in a 2 to 1 molar ratio, pure L-PE or L-PE:CEBA in a 6 to 4 molar ratio.

Cellular transfection: For transfection of cells, 2 x 10<sup>6</sup> cells in 4 ml of serum-free medium were plated in 100 25 mm plastic petri dishes (Falcon, Oxnard, CA). The plasmid DNA-liposome complex was prepared by first adding 1) DNA and then 2), liposomes and mixing gently. The complex was then suspended in 1 ml of serum-free medium and added to the cells. Four hours later, the cells were 30 washed twice, resuspended in 10 ml of serum-containing medium, and subsequently harvested, 44 hours later. Just prior to harvesting, the cells were washed 2 times, and the plates were then scraped with a rubber policeman. The cells were centrifugated at 1,000 x g for 5 min, and 35 0.135 ml of 0.25 M Tris buffer pH 7.5, 5 mM EDTA was

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added to each pellet. The cells were freeze-thawed 3x, heated at 65°C for 10 min, and spun at 12,500 x g for 10 min. The supernatant was assayed for protein and 20 µg of supernatant protein per sample was used to measure CAT activity, as described in Example 4.

Results: The results demonstrate the ability of cationic liposomes to mediate high level transfection of two different human small cell lung cancer lines (H69 and H82) and a squamous cell lung cancer line (H520). All three lines were very efficiently transfected by RSV-CAT when complexed to 3 different cationic liposome formulations (Figure 21). These human cell lines were transfected either as, or more efficiently than rodent tumor cell lines transfected under comparable conditions.

15

Example 12Transfection of lung cancers in mice by intravenous injection of cationic lipid carrier:DNA complexes

Mouse: C57/black 6, female, 25 grams.

Cancer: B16, mouse melanoma line which is highly metastatic to lung. The cell line was grown in RPMI 1640 medium supplemented with 5% fetal calf serum.

Lipid carrier: DOTAP:Cholesterol=1.1, 10mM in 5% dextrose in water.

25 Plasmid: pZN20.

Ratio: Cationic lipid:DNA=6 nmoles:1 µg, 100 µg in 200 µl of 5% dextrose in water were injected by tail vein into each mouse.

Inoculation of cancer cell line into mice and administration of CAT expression plasmid-cationic lipid carrier complexes:

B16 cells were trypsinized off the plates and 50,000 cells were inoculated into each mouse by

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intravenous injection into the tail vein. Two weeks after injection, cationic lipid carrier-DNA complexes were injected via tail vein. Lungs were collected 48 hours postinjection, infused with 33% O.C.T., frozen in a dry ice-ethanol bath, cryosectioned, and processed for immunohistochemical analysis to detect intracellular CAT protein.

Immunohistochemical analysis: Procedure:

Organs were removed, appropriately trimmed, embedded in OCT and snap frozen. Frozen tissues were sectioned at 6  $\mu$ m, collected onto glass slides, fixed for 10 minutes in 4°C acetone and then placed in 0.2% Triton X-100 to permeabilize membranes. Sections were then incubated for 12-48 hours with the monoclonal anti-CAT antibody or isotype negative control antibody at the appropriate dilution. After washing, a biotinylated antibody directed against the primary antibody (Zymed, S. San Francisco) was added for a minimum of 60 minutes, followed by application of the streptavidin-peroxidase complex (Zymed) for 60 minutes and then application of the substrate-chromogen appropriate for the enzyme label used. Slides can then be counter-stained in dilute hematoxylin or left unstained and coverslipped in water-soluble mounting media for examination.

Results:

Immunohistochemical analysis are shown in Figure 8 and demonstrate that B-16 melanoma lung tumors (8A, indicated by arrows) as well as intravascular tumor emboli (8B, indicated by arrows) are efficiently transfected after iv injection of DNA-lipid carrier complexes. Both lung tumors and intravascular tumor emboli show intense staining, indicating efficient, a generalized transfection in vivo. Tumor bearing mice, which did not receive an injection of DNA-lipid carrier complexes, show no CAT activity in the lung or in any

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lung tumor cells (8C). The ability to transfect tumors present within mammalian hosts by systemic administration of a cloned gene has not previously been demonstrated.

Example 13

- 5     Demonstration of High Level CAT Gene Expression in Multiple Tissues After Intravenous (iv) Injection of pZN27 Alone, or  
          pZN27:DDAB:Cholesterol SUV Complexes.

- 10   Lipid carrier:     DDAB:Cholesterol=1:1, stock 10mM in 5% dextrose. After addition of 5% dextrose to the dried lipid film, the SUV were prepared by sonication in a bath sonicator for 20 minutes.

- 15   Plasmid:         pZN27.

DNA:Lipid carrier Ratio: Cationic lipid:plasmid DNA = 5 nanomoles:1 µg DNA.

DNA dose:

- pZN27 alone: Individual mice received 500 µg, 1  
20   mg, 2 mg, or 500 µg, followed 4 hours later by a second 500 µg dose, respectively of pZN27 in 200 µl 5% dextrose in water by tail vein injection.

- pZN27 complexed to lipid carriers: 100 µg plasmid DNA complexed to 500 nanomoles to DDAB:Cholesterol SUV  
25   lipid carriers in 200 µl 5% dextrose in water was injected by tail vein per mouse.

Mice: ICR, female, 25 grams.

- Tissue extraction procedure: Each organ was homogenized in 0.3 ml of 0.25 M Tris-HCl pH 7.8, 5 mM EDTA, and the  
30   resulting extract was centrifuged and the supernatant was then subjected to 3 cycles of freeze-thaw and then heated to 65°C for 20 min.

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CAT assay procedure: The protein concentration of each tissue extract was quantitated using a Coomassie Blue-based protein assay (BioRad, Richmond), and same amount of total protein from each tissue extract was added in the CAT assay, together with 10  $\mu$ l of 20 mM acetyl CoA+12  $\mu$ l of  $^{14}$ C-chloramphenicol (25  $\mu$ Ci/ml, 55 mCi/mole, Amersham)), at 37°C for 13 hrs.

Results:

Significant levels of CAT gene expression were seen in each of the 6 different tissues assayed (lung, heart, liver, kidney, spleen, and lymph nodes) after injection of either pZN27 alone, or pZN27 complexed to DDAB:Cholesterol lipid carriers. Expression of a transgene in multiple tissues in vivo after systemic injection of a naked expression plasmid previously has not been demonstrated.

Example 14

Induction of High Levels of Human Interleukin-2 in the Spleen

and Lymph nodes of Mice by Intravenous Injection of Cationic

Lipid carriers Complexed to a CMV-Interleukin-2 Gene

Mouse: C57/black 6, female, 25 grams.

Cancer: B16, mouse melanoma line which is highly metastatic to lung. The cell line was grown in RPMI-1640, 5% fetal calf serum.

Lipid carrier: DDAB:Cholesterol=1.1, 10 mM in 5% dextrose in water.

Plasmid: pZN46 (the HCMV promoter enhancer fused to the human interleukin-2 coding sequence).

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Ratio: Cationic lipid:DNA=5 nmoles:1  $\mu$ g  
DNA in 200  $\mu$ l of 5% dextrose in  
water administered per injection.

Inoculation of the tumor cell line into mice and

5 administration of a human interleukin-2 expression  
plasmid-cationic lipid carrier complexes:

B16 cells were trypsinized off the plates, and  
50,000 cells were inoculated into each mouse by  
intravenous injection into the tail vein. Starting 2  
10 days after the tumor cell injection, cationic lipid  
carrier-DNA complexes were injected via tail vein 2 times  
per week for a total of 2 weeks. The animals were  
sacrificed 2 weeks post tumor cell injection, the spleen  
and lymph nodes were removed, rendered into single cell  
15 suspensions using a tissue grinder, and then cultured for  
24 hours in RPMI-1640, 10% fetal calf serum in 100 mm  
plastic dishes in a 37°C incubator. After 24 hours the  
supernatant was collected and the concentration of human  
interleukin-2 in the supernatant was determined using a  
20 human IL-2 ELISA.

Results:

One hundred picograms of human IL-2/ml per ml was  
present in the spleen cell supernatant and 91 pg/ml IL-2  
was present in the lymph node cell supernatant from the  
25 mouse which was injected with the pZN46-DDAB:Cholesterol  
lipid carrier complexes. No human IL-2 was detected in  
either spleen cell or lymph node cell supernatants  
derived from mice which received an identical injection  
of B-16 melanoma cells, but which did not receive the  
30 pZN46-DDAB:Cholesterol lipid carrier complexes. Thus,  
substituting the human IL-2 gene coding region for the  
CAT gene coding region in a HCMV expression plasmid  
resulted in high level expression of the IL-2 gene in  
vivo and the production of large amounts of a secreted  
35 human IL-2 protein in a mouse.



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Example 15Induction of High Level Expression of the Human CFTR genein Mice Treated by iv Administration of pZN32:Cationic5 Lipid carrier ComplexesMice: ICR female, 25 gramsLipid carrier: DDAB:Cholesterol=1:1 SUV, 10 mM in 5% dextrose in waterPlasmid: pZN32 (the HCMV promoter enhancer fused to the  
10 human CFTR coding sequence)Ratio: Cationic lipid:DNA=5 nmoles:1 $\mu$ g plasmid DNA.Dose: A total of 100  $\mu$ g of plasmid DNA in 200  $\mu$ l of 5% dextrose in water administered per iv tail vein injection.15 Procedure:

Forty-eight hours after injection the mice were sacrificed, the lungs were removed and trimmed, embedded in OCT, and snap frozen. Frozen tissues were sectioned at 6  $\mu$ m, collected onto glass slides, and the fixed for  
20 10 minutes in 4% acetone. Immunolocalization of CFTR was then performed using the affinity purified rabbit polyclonal anti-CFTR antibody,  $\alpha$ -1468 (Cohn, et al., (1991) *Biochem. Biophys. Res. Comm.* 181:36-43). The procedure used was identical to the one described in  
25 Marino et al., (1991) *J. Clin. Invest.* 88:712 with the following variation. After washing, a biotinylated antibody directed against the rabbit polyclonal antibody (Zymed) was added for 60 minutes, followed by application of the streptavidin phosphatase complex (Zymed) for 60  
30 minutes and then application of the substrate-chromogen. Slides were then coverslipped in water-soluble mounting media for examination.

Results:

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Photomicrographs of frozen sections (viewed at different magnifications) of mouse lung 48 hours following iv injection to pZN32-DDAB:Cholesterol (1:1) liposome complexes and lung from untreated control are shown in Figures 12A-E. As demonstrated by the intense staining with the polyclonal anti-CFTR antibody,  $\alpha$ -1468, the overwhelming majority of the airways were transfected with the human CFTR gene. In 12A, 12C and 12E. by visual inspection, essentially all the cells in transfected airways stain positively, demonstrating that the overwhelming majority of airway cells are transfected with the human CFTR gene in vivo with a single iv dose of pZN32 complexed to DDAB-cholesterol (1:1) liposome-treated and control animals could not be distinguished histologically. Significant expression of the human CFTR gene is present in at least 50% of all the airways and at least 50% of all of the airway lining cells (by visual inspection) in mouse lungs for at least 60 days following a single iv dose of pZN32 complexed to DDAB-Cholesterol (1:1) liposomes. Frozen sections of mouse lungs from control animals (12B and 12D) do not show any detectable staining for CFTR, confirming that all the CFTR expression present in 12A, 12C and 12E is due to transfection of lung cells with the human CFTR gene.

As shown by the above results, a single iv dose of an expression construct, containing a gene of interest, complexed to cationic liposomes transfects the majority of the cells lining the conducting airways of the lung, the gene product is present in the lung for at least 60 days, the expression appears to be airway cell-specific, and there is no histological evidence of damage following exposure. This is important because liposomes are well tolerated and non-immunogenic. Furthermore, the appearance, behavior, and life span of mice treated with intravenously injected pZN32:DDAB-Cholesterol (1:1)

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complexed appear normal, and are indistinguishable from untreated, normal control animals. This lack of toxicity, as demonstrated by analysis of appearance, behavior, life span and detailed histologic analysis of a wide variety of tissues from these animals demonstrates the lack of toxicity produced by *in vivo* delivery of pZN32-DDAB:Cholesterol complexes. Additionally, the effects of repeated *iv* administration of the DNA/liposome complexes is effective and is non-toxic. The cationic liposome-mediated DNA delivery by *iv* injection provides high level, lung-specific transgene expression *in vivo*.

#### Example 16

##### Demonstration of CAT Gene Expression in Lung and Liver

##### After Intravenous Injection of Different CAT gene-Containing Plasmids

Lipid carrier: DDAB:Cholesterol= 1:1, stock 5mM in 5% dextrose in water.

Plasmids: Plasmids are indicated below.

DNA-Lipid carrier Ratio: Cationic lipid: plasmid DNA = 1 nanomole: 1  $\mu$ g

Dose: 100  $\mu$ l DNA in 200  $\mu$ l volume injected intravenously by tail vein injection.

Mice: ICR, female, 25 g

Procedure: The animals were sacrificed 24 hours after injection. The tissue extraction procedure and CAT assay were as described in Example 4 except that the CAT assay was incubated for 3 hr at 37°C and 2.0 mM paraoxon (Lai, et al., (1988) *Carcinogenesis* 9:1295-1302) was added to the liver samples. The results are shown in Figure 15. Lanes 1-12 are lung samples, lanes 13-24 are liver samples. Lanes 1, 2, 13, 14 are pZN51; lanes 3, 4, 15, 16 are pZN60; lanes 5, 6, 17, 18 are pZN61; lanes 7, 8,

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19, 20 are pZN62; lanes 9, 10, 21, 22 are pZN63; and lanes 11, 12, 23, 24 are pZN27.

Results:

pZN51, which does not contain an intron, is expressed as well as, or better than, plasmids containing an intron either 3' or 5' to the coding sequence.

Example 17

Generalized versus tissue and cell type-specific CAT gene expression produced by iv injection of CMV-CAT-liposome or CFTR-CAT-liposome complexes, respectively.

Mouse: ICR female, 25 grams.

Liposome: DDAB:Cholesterol=1:1 SUV, 10mM in 5% dextrose in water.

Plasmid: 1) pZN27 or 2) pBE3.8CAT (see Chou et al., (1991) J. Biol Chem 266:24471-24476, for construction).

Experimental conditions: Mice in groups of 3 received either (a), no treatment, or (b), a single iv tail vein injection of DDAB:Cholesterol liposomes complexed to 100 µg of) a 3.8 kb sequence of the 5' upstream region of the human CFTR gene fused to the CAT gene (pBE3.8CAT) or (c) pZN27. Mice were sacrificed 24 hours later and CAT activity assayed in lung, liver, spleen, lymph nodes, kidney and heart, as described in Example 4.

Immunohistochemical analysis of lung section from each of the groups was performed as described in Example 3.

Results:

Immunohistochemical staining of frozen lung sections from these mice showed that iv injection of CMV-CAT-liposome complexes produced high levels of red staining, indicating CAT gene expression in endothelial, alveolar and airway cells within the lung (16A). In contrast, CFTR-CAT-liposome complexes produced CAT gene

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expression primarily localized in airway epithelial cells (16B). This approximates the pattern of endogenous CFTR gene expression in rat lung, as determined by *in situ* hybridization studies (Trezise and Buchwald, (1991) Nature 353:434-437. A lung section from an uninjected mouse does not show red staining, indicating that CAT gene expression is present only in transfected cells (Fig. 16C). Figure 16D is a high magnification photomicrograph of alveoli from a CMV-CAT treated mouse and shows a high level of CAT gene expression in both alveolar cells and lung endothelial cells. A high magnification photomicrograph of alveoli from CFTR-CAT treated mice (Figure 16E) shows no significant CAT gene expression in either alveolar or endothelial cells, demonstrating that the CFTR promoter targets transgene expression to airway epithelial cells. This is the first demonstration that transgenes can be expressed within mouse lung in either a generalized or cell type-specific fashion depending on the regulatory element used, after iv injection.

CAT assay demonstrated that CMV-CAT produced significant CAT gene expression in the lung, liver, heart, spleen, lymph nodes and kidney, whereas CFTR-CAT produced lung-specific gene expression. Photographs of autoradiographic analysis of each tissue are shown in Figure 16 (F-K). Thus, the CMV promoter induces expression of a linked gene in a wide range of tissues, whereas the 5' flanking region of the human CFTR gene directs tissue-specific transgene expression after iv, liposome-based administration.

#### Example 18

Comparison of transfection focusing on iv injection of plasmid

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alone with iv injection of plasmid complexed to a lipid carrier.

Demonstration of widespread, high level CAT gene expression in in vivo after intravenous (iv) injection of  
5 pZN27 alone.

Plasmid: pZN27.

DNA:Liposome Ratio: Plasmid DNA alone, without liposomes, was injected.

DNA dose: 1 mg plasmid DNA in 200  $\mu$ l 5% dextrose  
10 in water was injected times 2 over a 4 hour period by tail vein per mouse. Mice were sacrificed 24 hours later and 17 different tissues were assayed for CAT gene activity.

Mice: ICR, female, 25 grams.

15 Tissue extraction procedure: Each organ was homogenized in 0.3 ml of 0.25 M Tris-HCl pH 7.8, 5 mM EDTA, and the resulting extract was centrifuged and then subjected to 3 cycles of freeze-thaw and then treated to 65°C for 20 min.

20 CAT assay procedure: The protein concentration of each tissue extract was quantitated using a ninhydrin-based protein assay (BioRad, Richmond), and the same amount of total protein from each tissue extract was added in the CAT assay, together with 10  $\mu$ l of 20 mM  
25 acetyl CoA+12  $\mu$ l of  $^{14}$ C-chloramphenicol (25  $\mu$ Ci/ml, 55 mCi/mole, Amersham), at 37°C for 13 hrs.

Results: An autoradiograph of this experiment is shown in 19A. Compared to control levels (lane 1), iv  
injection of pZN27 alone produced highly significant  
30 levels of CAT gene expression in the following tissues: lung, thymus, esophagus, heart, liver, spleen, stomach, small intestine, caecum, ovary, vagina, skeletal muscle, pancreas and lymph nodes. Thus, iv injection of the

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pZN27 expression plasmid alone can efficiently transfect a very large and diverse number of tissues in the body.

Demonstration of widespread, high level CAT gene expression in vivo after intravenous (iv) injection of  
5 pZN27 complexed to DDAB:Cholesterol (1:1) liposomes.

Plasmid: pZN27.

Liposome: DDAB:Cholesterol=1:1, stock 10 mM in 5% dextrose.

DNA:Liposome Ratio: liposome:plasmid 5 nmoles:1

10  $\mu$ g

DNA dose: 100  $\mu$ g plasmid DNA in 200  $\mu$ l 5% dextrose in water was injected by tail vein per mouse. Mice were sacrificed 24 hours later and 17 different tissues were assayed for CAT gene activity.

15 Mice: ICR, female, 25 grams.

Tissue extraction procedure: Each organ was homogenized in 0.3 ml of 0.25 M Tris-HCl pH 7.8, 5 mM EDTA, and the resulting extract was centrifuged and then subjected to 3 cycles of freeze-thaw and then treated to  
20 65°C for 20 min.

CAT assay procedure: The protein concentration of each tissue extract was quantitated using a ninhydrin-based protein assay (BioRad, Richmond), and same amount of total protein from each tissue extract was added in  
25 the CAT assay, together with 10  $\mu$ l of 20 mM acetyl CoA+12  $\mu$ l of  $^{14}$ C-chloramphenicol (25  $\mu$ Ci/ml, 55 mCi/mole, Amersham), at 37°C for 13 hrs.

Results: An autoradiograph of this experiment is presented in Figure 19B. Compared to control levels  
30 (lane 1), iv injection of pZN27:DDAB:Cholesterol complexes produced high levels of CAT gene expression in the following tissues: lung, thymus, esophagus, heart, liver, spleen, stomach, small intestine, large intestine, caecum, uterus, ovary, vagina, skeletal muscle, pancreas

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and lymph nodes. Thus, iv injection of pZN27:DDAB:Cholesterol complexes can efficiently transfect a very large and diverse number of tissues in the body.

5

Example 19IV Injection Of GM-CSF Expression Plasmid-Cationic LiposomeComplexes Produces Significant Antitumor Effects.

- Mouse: C57/black 6, female, 25 grams.
- 10 Cancer: B16, mouse melanoma line which is highly metastatic to lung. The cell line was grown in RPMI-1640, 5% fetal calf serum.
- Liposome: DDAB:Cholesterol=1:1, 10 mM in 5% dextrose in water. Twenty five thousand
- 15 B-16 cells were injected iv by tail vein.
- Plasmid: pZN84 (the HCMV promoter enhancer fused to the murine GM-CSF coding sequence as shown in Figure 17).
- 20 Ratio: Cationic lipid:DNA=5 nmoles:1  $\mu$ g. A total of 100  $\mu$ g of plasmid DNA in 200  $\mu$ l of 5% dextrose in water administered per injection.

Experimental outline: Mice in groups of eight received a

25 single iv tail vein injection of  $2.5 \times 10^4$  B-16 melanoma cells. Group 1 received no treatment, group 2 received bi-weekly injections of 100  $\mu$ g of pZN84 complexed to DDAB:Cholesterol liposomes, beginning 4 days prior to tumor cell injection and continuing for 2 weeks following

30 tumor injection. All mice were sacrificed 3 weeks following tumor cell injection and surface lung tumor nodules which were black and macroscopic were counted using a dissecting microscope.



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Results:

Control animals had  $64.5 \pm 19.7$  (S.E.M.) nodules per lung. In contrast, the GM-CSF-liposome-treated animals had  $11.9 \pm 3.6$  tumor nodules per lung ( $p < 0.01$  versus the control group, as assessed by Student's t test). Thus, iv injection of a cytokine gene produced a highly significant antitumor effect. This is the first demonstration that iv injection of a gene can produce anti-tumor activity *in vivo*.

10

Example 20

Prolonged, high level murine GM-CSF gene expression in vivo

after intravenous (iv) injection of pZN84 complexed to DDAB:Cholesterol (1:1) liposomes into goats.

15 Animals: Goats: female, 110 pounds

Plasmid: pZN84

Liposome: DDAB:Cholesterol=1:1, stock 10mM in 5% dextrose

DNA:Liposome Ratio: Liposome:plasmid=5:1

20 DNA dose: 1.0 mg plasmid DNA in 5 ml 5% dextrose in water was injected by jugular vein per goat.

Blood Sampling: Blood was drawn just prior to injection of pZN84 liposome complexes, and at 12, 24, 48, 72, 168 and 840 hours post-injection.

25 Murine GM-CSF ELISA assay procedure: Murine GM-CSF was measured in these serum samples using a commercial murine GM-CSF ELISA kit provided by Endogen. There is no cross reactivity between goat and murine GM-CSF by ELISA.

Results: As shown in Figures 22 and 23 (Goats A, B and 30 C), as compared to control levels, iv injection of pZN84:DDAB:Cholesterol complexes produced high sustained circulating levels of mouse GM-CSF protein for at least 168 hours following injection. However, by 840 hrs (35 days) GM-CSF levels were substantially reduced (Figure

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27, Goat A). No mouse GM-CSF protein was detected in the circulation of a goat which received 1 mg of the CAT gene (pZN51) complexed to DDAB:Cholesterol liposomes by the identical iv injection protocol. The goat receiving the CAT gene did express CAT antigen on the majority of its circulating white blood cells at 24 hours after injection, as determined by immunostaining for CAT antigen. No CAT antigen was detected in the white blood cells of the goats which were injected with GM-CSF gene-liposome complexes. Thus, iv injection of pZN84:DDAB:Cholesterol complexes can produce high level expression of the murine GM-CSF gene for prolonged periods in goats. IV injection of pZN51:DDAB:Cholesterol complexes can produce high levels of the CAT gene product in the majority of all circulating white blood cells in goats.

#### Example 21

##### Influence of liposome-GM-CSF plasmid complex on the course of

##### experimental viral pneumonia in mice.

Endogenous cytokines are believed to be included in the host response to a viral infection. It is therefore of interest to determine whether increased expression of a single cytokine, GM-CSF would influence the time course of viral pneumonia. The effect of delivering the murine GM-CSF plasmid-liposome complex via two different routes on the host response to a Sendai virus challenge is tested. The plasmid-liposome complex is delivered iv to one group of mice, and intranasally (in) to a second group. Dose, time of delivery, virus challenge dose and endpoints are the same for both groups. Dose of plasmid-liposome complex is 100

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µg/mouse/dose. Dosing for both protocols occurs on the following scale:

<u>Group</u>	<u>Initial PL* dose</u>	<u>2nd PL dose</u>	<u>Virus</u>
<u>challenge</u>	<u>Endpoints</u>		
5 IV Route	day-3	day 0	day 0
days 3, 7, 10			
IN Route	day-3	day 0	day 0
days 3, 7, 10			

\*PL - plasmid-liposome

- 10 Each experimental group consists of the following:  
 murine GM-CSF plasmid-liposome + virus: 3  
 mice

CAT plasmid-liposome + virus: 3 mice

Untreated + virus: 3 mice

- 15 This results in 9 mice per timepoint (27 total for each experiment, 54 total mice). All animal procedures are performed via NIH and AAALAC guidelines including use of appropriate analgesia.

- 20 Virus challenge is administered intranasally with Sendai virus strain 771076, a strain known to be pathogenic in mice. Endpoints include the following (these have been shown to be useful endpoints for Sendai pneumonia):

1. lung weight vs. body weight and brain weight
- 25 2. lung histopathology
3. quantitative virus recovery
4. Sendai specific antibody response
5. lung GM-CSF
6. lung TNF

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Example 22

High level expression of the CAT gene in mouse brain  
produced by injection  
of DNA alone or DNA-cationic liposome complexes directly  
5 into the

central nervous system.

Mice: ICR, female, 25 grams

Plasmid: pCIS-CAT

Liposome: DOTMA:DOPE (1:1)

10 For each mouse injected, 2.5  $\mu$ g DNA was diluted in 5%  
dextrose, then mixed with liposomes diluted to the same  
volume in 5% dextrose. Five microliters were injected  
stereotactically into the right ventricle of each mouse.

Ratio: Plasmid:lipid = 1:0 (1 mg DNA: 0  
15  $\mu$ moles DOTMA)

Plasmid:lipid = 1:1 (1 mg DNA: 1  $\mu$ moles  
DOTMA)

Plasmid:lipid = 1:3 (1 mg DNA: 3  $\mu$ moles  
DOTMA)

20 Plasmid:lipid = 1:4 (1 mg DNA: 4  $\mu$ moles  
DOTMA)

Plasmid:lipid = 1:6 (1 mg DNA: 6  $\mu$ moles  
DOTMA)

Mice were sacrificed 48 hr. post injection.

25 Brains were removed and separated into left and right  
hemispheres. Each hemisphere was homogenized in 250  $\mu$ l  
0.25 M Tris pH 7.8, 5 mM EDTA followed by three cycles  
of freeze-thaw then 10 min. at 65°C.

The amounts of extracts used for CAT assay were  
30 normalized to protein levels. 0.3 ml of  $^{14}$ C-  
chloramphenicol was used for each assay. Assays were  
carried out at 37°C overnight. The reaction products  
were separated on TLC plate and exposed to film, as  
described in Example 4.

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Results: These results demonstrate that high level expression of a heterologous gene can be produced throughout the brain by injection of DNA-liposome complexes (at appropriate ratios) directly into the central nervous system. They also demonstrate that injection of an expression plasmid alone can produce significant transgene expression in the brain. The results are shown in Figure 20.

Example 23

10 Demonstration of CAT gene expression in the lung after intravenous (iv) injection of pRSV-CAT:L-PE:CEBA complexes.

Lipid carrier: L-PE:CEBA=1:1, stock 20 mM in lipid carrier buffer.

15 Plasmid: pRSV-CAT.

DNA:Lipid carrier Ratio: Lipid carrier:plasmid=1 nanomole cationic lipid: 1 µg plasmid DNA.

DNA dose: 100 µg plasmid DNA in 200 µl 5% dextrose in water was injected by tail vein per mouse.

20 Mice: Balb/c, female, 25 grams.

Tissue extraction procedure: Forty eight hours after tail vein injection, the animals were sacrificed, whole lung was homogenized in 1ml of 0.25M Tris-HCl pH 7.8, 5mM EDTA, 80 µg/ml PMSF and the resulting extract was centrifuged and then the supernatant was subjected to 3 cycles of freeze-thaw and then heated to 65°C for 20 min..

CAT assay procedure: 100 µl of extract+10 µl of 20 mM acetyl CoA+4 µl of <sup>14</sup>C-chloramphenicol (25 µCi/ml, 55 mCi/mmol, Amersham) were incubated together at 37°C for 6 hr. At 3 hours, an additional 10 µl of acetyl CoA was added.

Results:

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This experiment showed that a significant level of CAT activity (indicative of expression of the transgene) was present in the lung of the animal injected with lipid carrier:DNA complexes, but not present in the lungs from 5 control animals.

#### Example 24

Demonstration of CAT gene expression in multiple tissues after intravenous (iv) injection of pZN20-CAT:DDAB:DOPE complexes.

10        Lipid carrier: DDAB:DOPE=1:1, stock 10mM in 5% dextrose.

Plasmid: pZN20.

DNA:Lipid carrier Ratio: Lipid carrier:plasmid=(A) 3 nmole cationic lipid:

15        1 µg plasmid DNA (SUV). (B) 6 nmole cationic lipid: 1 µg plasmid DNA (MLV).

DNA dose: 100 µg plasmid DNA in 200 µl 5% dextrose in water was injected by tail vein per mouse. Three mice each received this dose of MLV:pZN20 and 3

20 mice each this dose of SUV:pZN20.

Tissue extraction procedure: Each organ was homogenized in 0.3 ml of 0.25 M Tris-HCl pH 7.8, 5 mM EDTA, and the resulting extract was centrifuged and then the supernatant was subjected to 3 cycles of freeze-thaw

25 and then heated to 65°C for 20 min..

CAT assay procedure: The protein concentration of each tissue extract was quantitated using a Coomassie Blue-based protein assay (BioRad, Richmond, CA), and the same amount of total protein from each tissue extract was

30 added in the CAT assay, together with 10 µl of 20 mM acetyl CoA+12µl of <sup>14</sup>C-chloramphenicol (25 µCi/ml, 55 mCi/mole, Amersham), at 37°C for 13 hrs.

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Results:

This experiment demonstrated that iv injection of pZN20:DDAB:DOPE complexes gave significant levels of CAT gene expression in each of 6 different tissues including lung, heart, liver, spleen, kidney and lymph nodes. Furthermore, MLV lipid carriers mediated equal or higher levels of *in vivo* transgene expression than did SUV lipid carriers composed of the same lipids.

Example 2510                   Production of Vaccines

The subject invention may find general utility in the production of vaccine, *in vivo*, for a variety of diseases. It has been shown that chimeric, genetically-engineered proteins of antigen fragments linked to granulocyte-macrophage colony-stimulating factor (GM-CSF), augment antigen presentation and increase immune response to the antigen when injected *in vivo* (Tao and Levy, (1993) *Nature* 362:755-758. Thus a nucleic acid sequence coding for a particular antigen may be fused to the nucleic acid coding for GM-CSF and expressed in appropriate cells *in vivo* using this invention. This approach could dramatically improve the control of proliferation of malignant cells in the treatment of cancer. Other uses would include *in vivo* vaccine production against a variety of viral and other infectious diseases, particularly those diseases in which an immune response has been unsuccessful or weak by conventional vaccine strategies. Of particular interest would be those diseases, such as HIV, which are characterized by proteins with hypervariable domains. Nucleic acid sequences encoding a variety of possible hypervariable domain sequences could be fused to GM-CSF and expressed simultaneously *in vivo* to elicit an immune response against many different strains of the virus.

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Example 26Treatment of Tumor Proliferation

- Nucleic acid sequences such as those coding for protein 53 may be useful in treating tumor proliferation.
- 5 Sequences coding for the tumor suppressor protein p53 may be expressed intracellularly *in vivo* using appropriate expression vector-lipid complexes. Such complexes can be conveniently targeted to tumor cells by appropriate antibodies or ligands conjugated to the lipid complexes.
- 10 Initial information regarding transfection of cells is developed using cultured cells, such as the tumor cell line K562. Once p53 gene expression is verified in K562 cells and the optimal vector have been identified, the following *in vivo* anti-tumor experiment is conducted.
- 15 SCID mice receive approximately 25 million K562 cells per mouse by intraperitoneal injection. Approximately three (3) weeks after injection, tumor-bearing mice in groups of ten (10) receive the following: no treatment/control group; p53 vector liposome complexes by intravenous
- 20 injection; p53 vector liposome complexes by intraperitoneal injection; reported gene vector liposome complexes. The anti-tumor effects of the expression of p53 is then be assessed based upon survival of the mice.

Example 27

- 25 Erythropoiesis, the production of red blood cells, can cause continuously throughout the life span to offset cell destruction. Erythropoiesis is a very precisely controlled physiological mechanism and having sufficient numbers of red blood cells to be available in the blood
- 30 for proper tissue oxygenation, but not so many that the cells would impede circulation. The formation of red blood cells occurs in the bone marrow and is under the control of the hormone erythropoietin. Because of erythropoietin is essential in the process of red blood



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cells formation, the hormone has potential use for application in both the diagnosis of the treatment of blood disorders characterized by low or defective red blood cell production. Gene therapy using the coding  
5 sequence for erythropoietin does find use in a variety of disease states, disorders and states of hematologic irregularity including anemia, in particularly correction of anemia of a type associated with chronic renal failure and the like. A nucleic acid sequence coding for a  
10 polypeptide having EPO activity is inserted into an appropriate transcription or expression cassette and introduced into a host mammal as naked DNA or complexed with an appropriate liquid carrier. Monitoring of the production of active EPO polypeptide can be performed as  
15 described for example in USPN 4,703,008.

As shown by the above results, a plurality of tissues can be transformed following systemic administration of transgenes, either complexed to a lipid carrier or as naked nucleic acid. Expression of  
20 exogenous DNA following intravenous injection of a cationic lipid carrier/exogenous DNA complex into a mammalian host has been shown in multiple tissues, including T lymphocytes, metastatic tumors and intravascular tumor emboli. Expression of exogenous DNA  
25 in multiple different tissues, including those of the reticuloendothelial system has been obtained following intravenous injection of an expression plasmid as naked DNA. The ability to transfect T lymphocytes *in vivo* will have a dramatic impact on the treatment of AIDS, cancer,  
30 multiple sclerosis, and arthritis. *In vivo* transfection of cardiac endothelial cells will have a dramatic impact on the treatment of heart disease and heart attacks. *In vivo* transfection of lung cells will have a dramatic impact on the treatment of cystic fibrosis, asthma,  
35 emphysema and lung cancer. *In vivo* transfection of bone

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marrow cells will have a dramatic impact on the treatment of cancer, blood diseases and infections.

The *in vivo* gene therapy delivery technology as described above is non-toxic in animals and transgene  
5 expression has been shown to last for at least 60 days after a single administration. The transgene does not appear to integrate into host cell DNA at detectable levels *in vivo* as measured by Southern analysis, suggesting that this technique for gene therapy will not  
10 cause problems for the host mammal by altering the expression of normal cellular genes activating cancer-causing oncogenes, or turning off cancer-preventing tumor suppressor genes. Additionally, transgene expression after systemic administration of a DNA expression vector  
15 alone has been shown; transgene expression was produced in the lung for at least three weeks after a single administration of a DNA expression vector without a carrier system.

Systematic injection of heterologous genes into  
20 adult animals can produce very high level transgene expression in a wide range of tissues, and transfect the majority (>70%) of all cells present in many of these tissues as demonstrated above. In contrast, previous studies attempting direct transfer of heterologous genes  
25 into adult animals have reported transfection limited to one or a few tissues, low levels of transgene expression in these tissues and (whenever histochemical analysis was included) transfection limited to less than 1% of the cells present in transfected tissues.

30 In addition to transfecting the majority of all cells present in the lung, using the methods and constructs described above, high level transgene expression has been obtained in a wide variety of other tissues and cell types. These include:

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- 5                   -    Transfection of the majority of all cells present in the spleen and lymph nodes, including transfection of greater than 70% of all the T lymphocytes. The ability to efficiently transfect T lymphocytes *in vivo* permitting for the first time specific molecular approaches to both anti-HIV therapy and to selective modulation of the immune response.
- 10                  -    Efficient transfection of visceral tumors and of intravascular tumor emboli after iv injection of DNA into tumor-bearing hosts. Previously, gene transfer studies involving cancer have been restricted solely to *ex vivo*
- 15                  -    approaches. Our work now permits direct transfection with transgenes which provide tumor suppressor, anti-oncogene and/or anti-metastases activity within tumors in tumor-bearing hosts.
- 20                  -    Transfection of the majority of cardiac vascular endothelial cells, as well as bone marrow-derived hematopoietic cells, including the great majority of blast cells present in the bone marrow, by systemic delivery of
- 25                  -    heterologous genes. These results create dramatic and new means for controlling ischemic heart disease and hematopoiesis at the molecular level.
- 30                  -    Demonstration of the ability to produce high level *in vivo* expression of a variety of biologically important transgenes, as exemplified by human CFTR, IL-2 and GM-CSF.

All publications and patent applications are herein incorporated by reference to the same extent as if  
35 each individual publication or patent application was

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specifically and individually indicated to be incorporated by reference.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many  
5 changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

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WHAT IS CLAIMED IS:

1. A complex of an expression cassette comprising:  
as operably joined components, a transcriptional  
5 and translational initiation regulatory region, a nucleic  
acid sequence of interest, and a transcriptional  
termination regulatory region, wherein said regulatory  
regions are functional in the cells of a mammalian host,  
and optionally, a cationic lipid carrier, and wherein  
10 said nucleic acid sequence of interest is free of  
introns.
2. A complex according to claim 1 wherein an  
intron is positioned 5' to said nucleic acid sequence.
3. A complex according to claim 1 or 2 wherein  
15 5' to said transcription and translational initiation  
region is at least one of an enhancer sequence and an  
intron.
4. A complex according to any one of the  
preceding claims wherein said transcriptional initiation  
20 regulatory region provides for tissue specific  
expression.
5. A complex according to any one of the  
preceding claims wherein said cationic lipid carrier is  
prepared from one or more lipids selected from the groups  
25 consisting of DDAB, DOTMA, DOPE, Cholesterol, and L-PE,  
DOTAP, and CEBA.
6. A complex according to any one of the  
preceding claims wherein said nucleic acid sequence of  
interest is CFTR, or interleukin 2.

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7. A complex according to claim 1 wherein the expression cassette is pCIS-CAT, pZN27, pZN51, pZN32, or pZN46.

8. A complex of a transcription cassette  
5 comprising: as operably joined components, a transcriptional initiation region, a nucleic acid sequence of interest, and a transcriptional termination regulatory region, wherein said regulatory regions are functional in the cells of a mammalian host, and  
10 optionally a cationic lipid carrier.

9. A complex according to claim 8, wherein said nucleic acid sequence of interest is an antisense sequence.

10. A pharmaceutical composition comprising:  
15 a complex according to any one of the preceding claims.

11. A complex according to any one of claims 1 to 9 or a composition according to claim 10 for use in a method of treatment of the human or animal body.

20 12. A complex or composition for use according to claim 11 wherein the method of treatment comprises the systemic introduction into the body of the complex or composition.

13. Use of an expression cassette as defined in  
25 any one of claims 1 to 7 or a transcription cassette according to any one of claims 8-9 for the manufacture of a medicament for the treatment of the human or animal body by systemic gene therapy.

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14. Use of a complex as defined in any one of claims 1 to 9 for the manufacture of a medicament for the treatment of the human or animal body by systemic gene therapy.

5        15. A non-human transgenic mammal wherein at least one cell type or tissue in addition to lung or liver contains a recombinant gene sequence introduced systemically into said mammal or an ancestor of said mammal.

10       16. A non-human transgenic mammal according to claim 15 wherein the recombinant gene sequence comprises an expression cassette as defined in any one of claims 1 to 7, or a transcription cassette as defined in any one of claims 8-9.

15       17. A non-human transgenic mammal according to claim 15 or 16 wherein said cell type or tissue is one or more cell type or tissue selected from the group consisting of spleen, lymph node, heart, kidney, bone marrow, and T-lymphocytes.

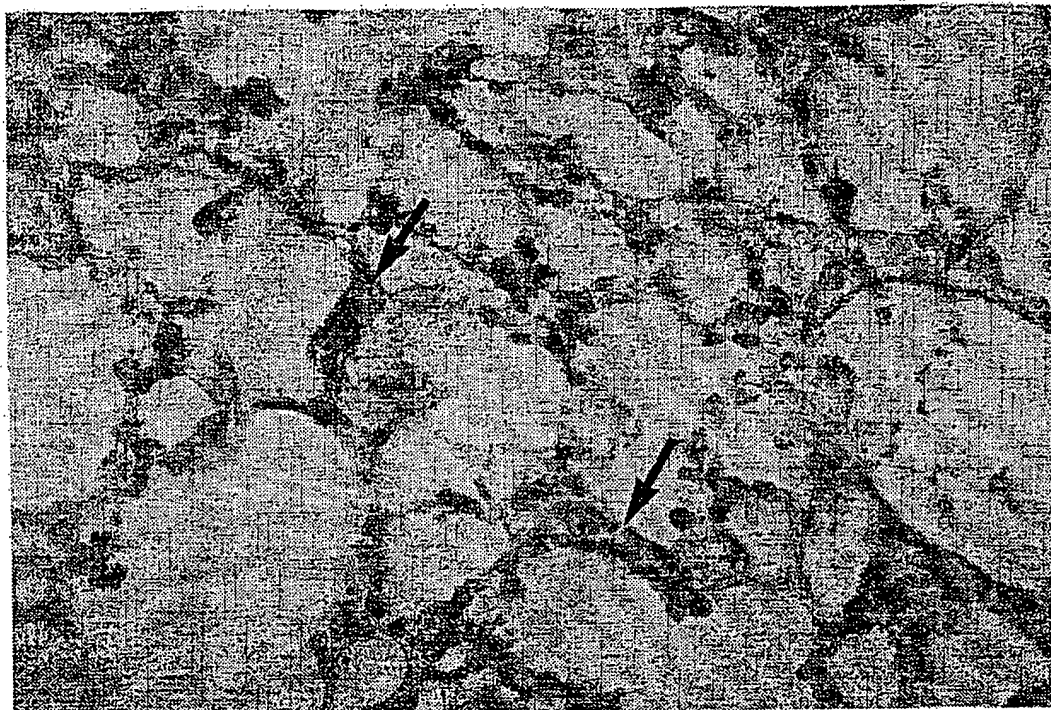


FIGURE 1A

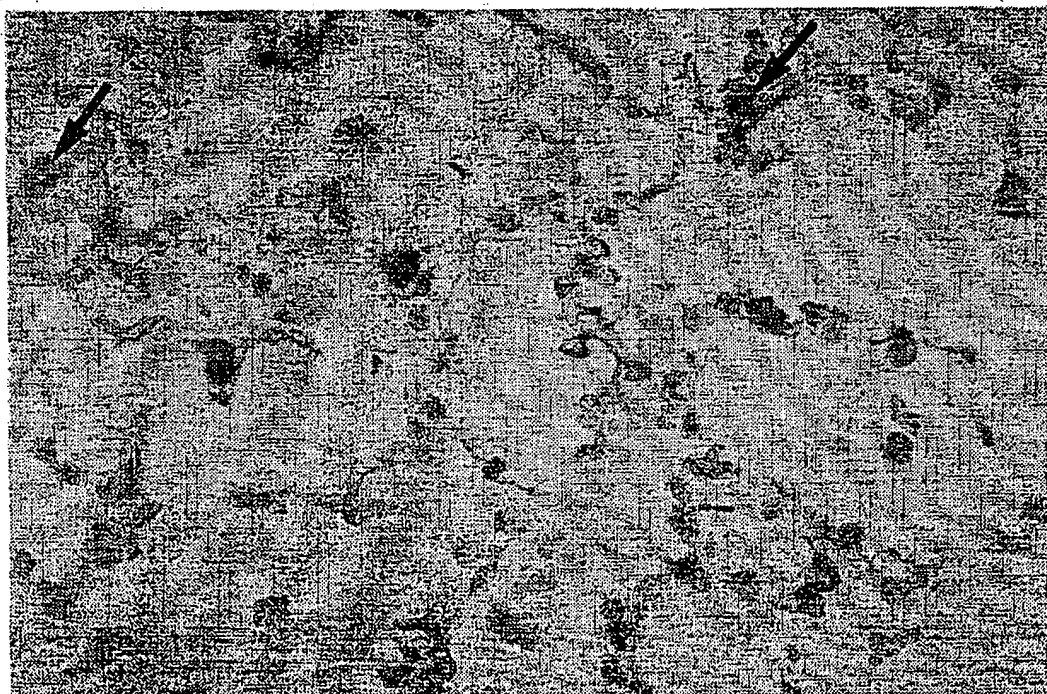
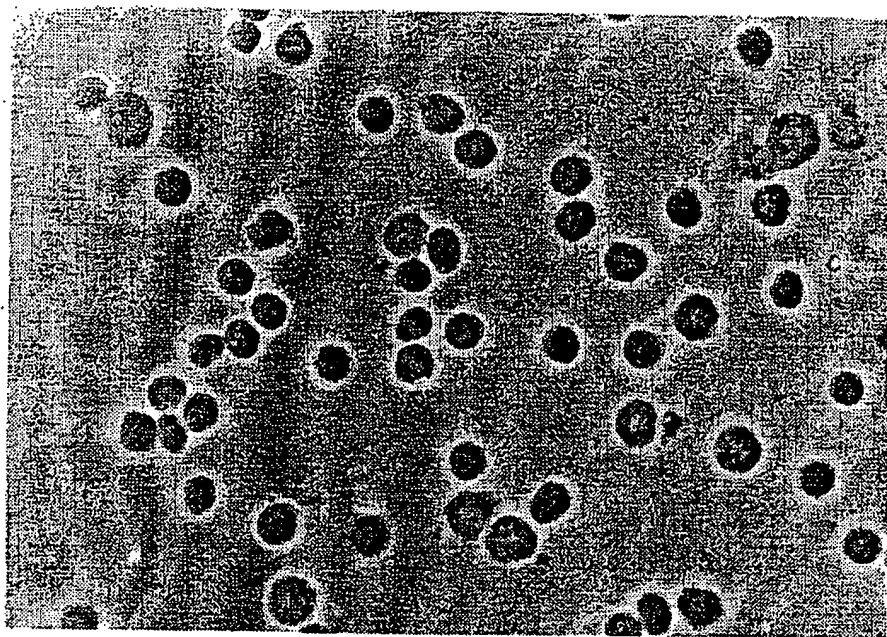


FIGURE 1B

Control

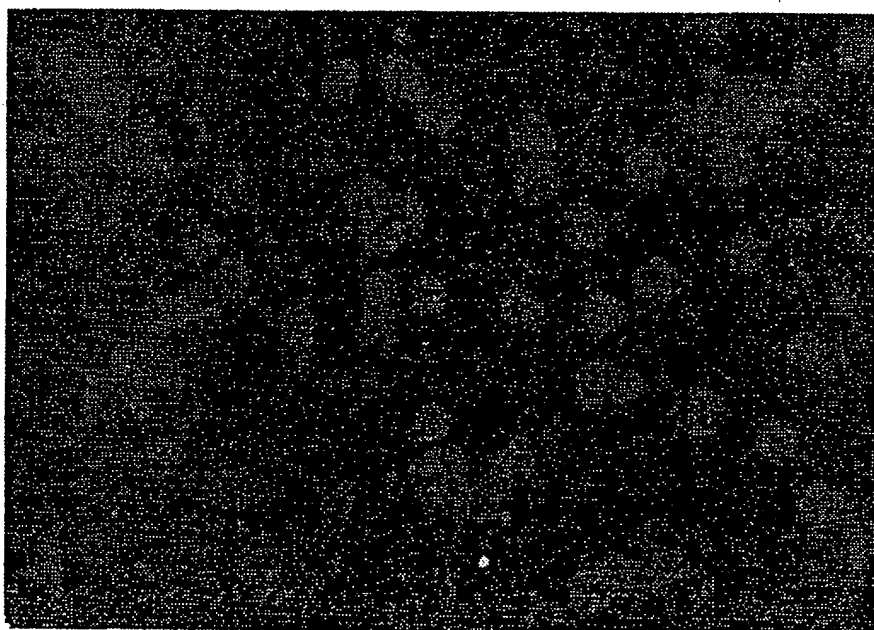


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Phase Contrast

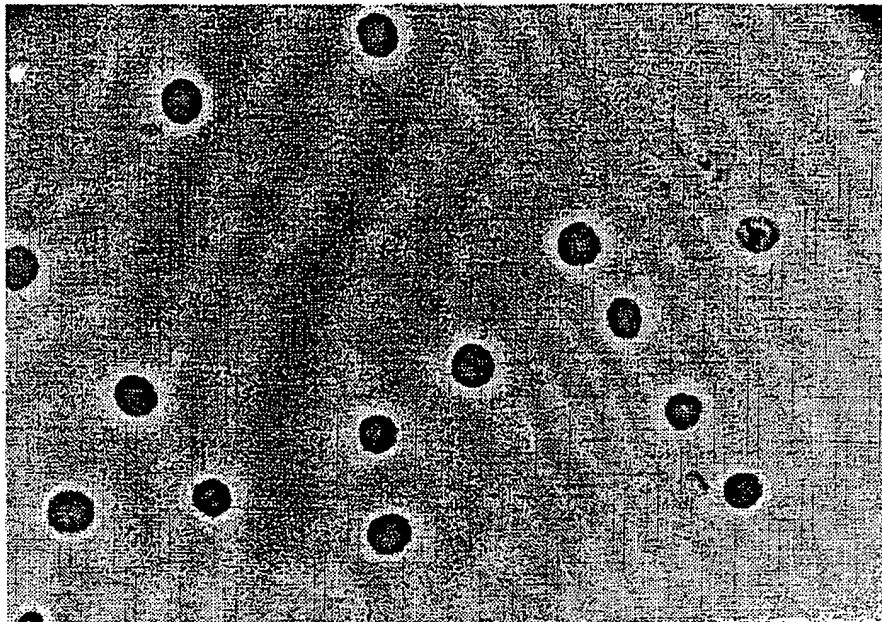
FIGURE 2A



Fluorescent

FIGURE 2B

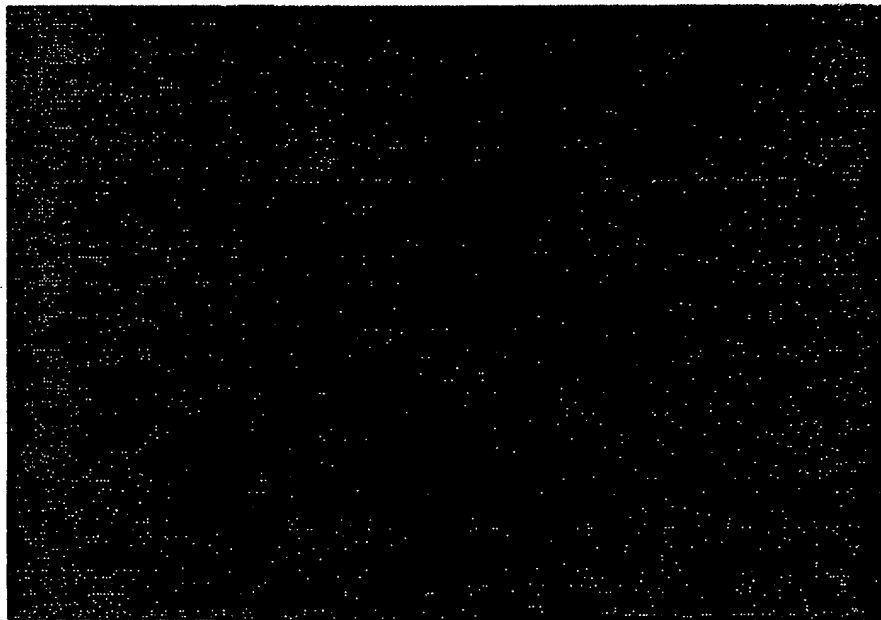
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Phase Contrast

FIGURE 2C

Control



Fluorescent

FIGURE 2D

Control

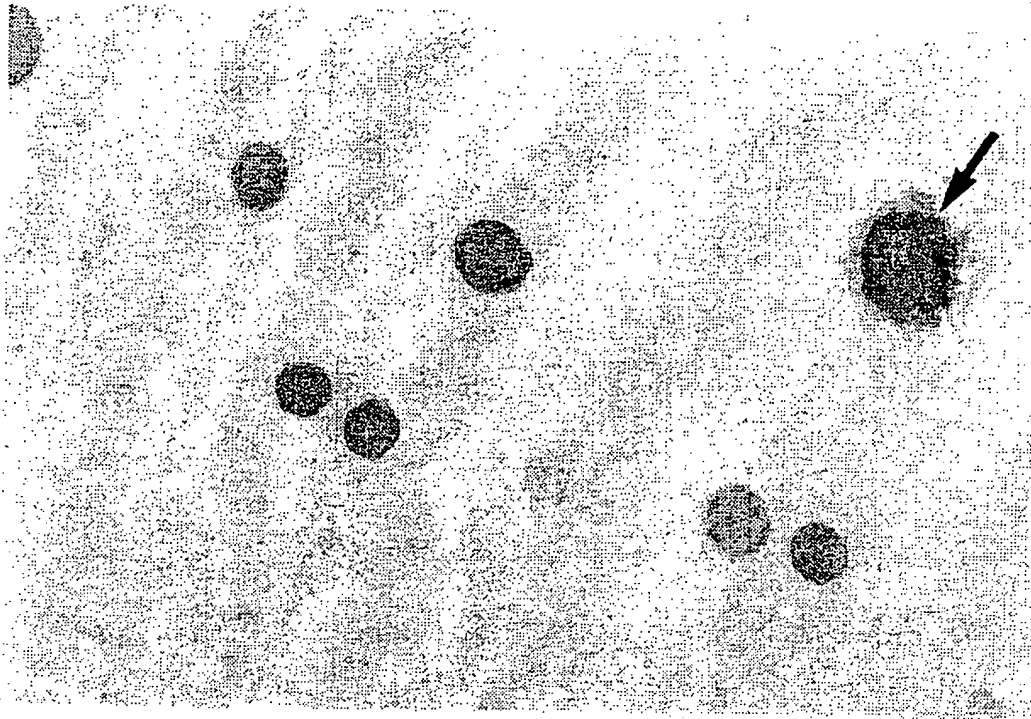


FIGURE 3A

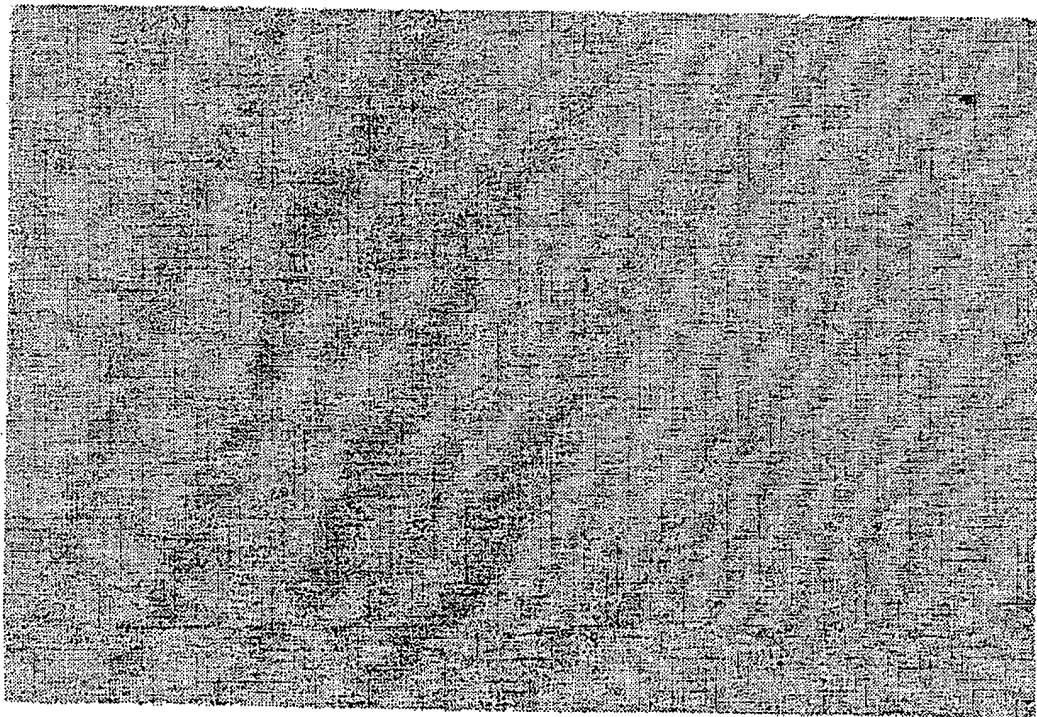
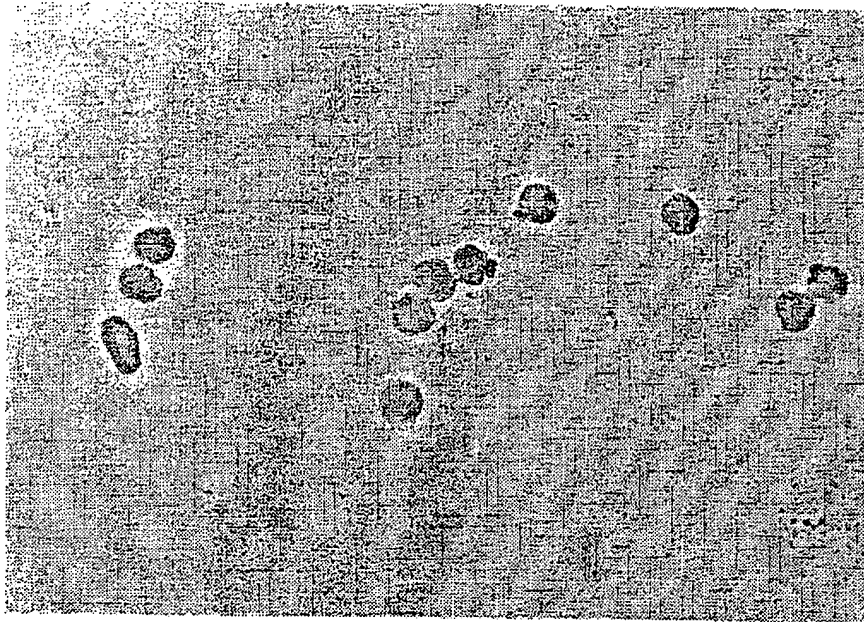


FIGURE 3B

Control

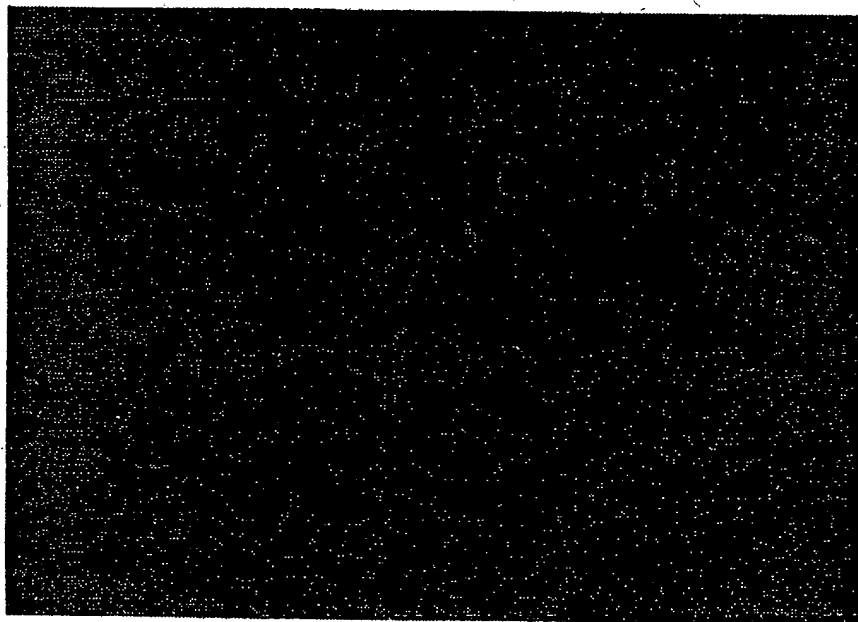
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Phase Contrast

FIGURE 4A



Fluorescent

FIGURE 4B

**SUBSTITUTE SHEET**

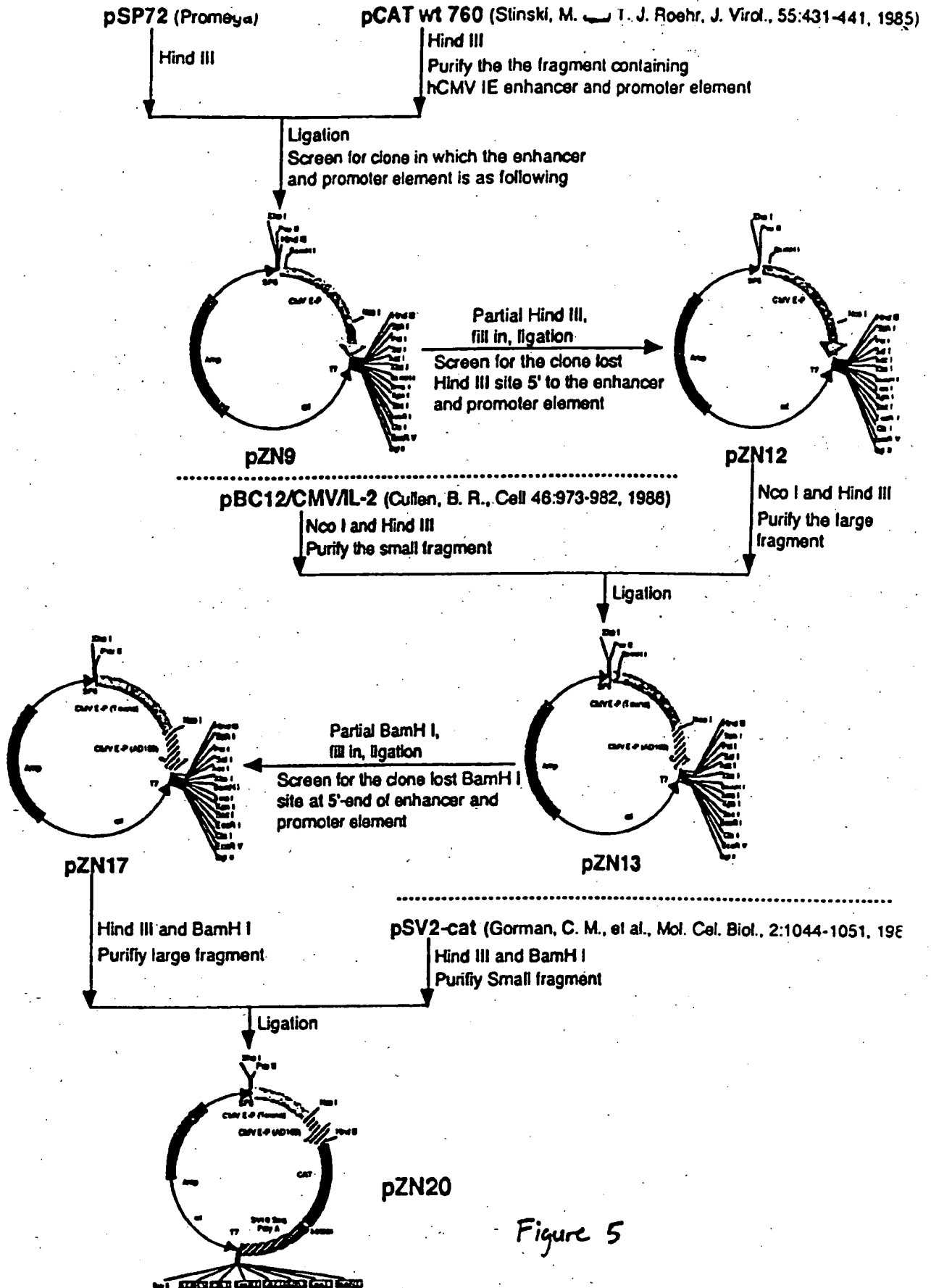


Figure 5

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Positions of Restriction Endonucleases sites (unique sites underlined)

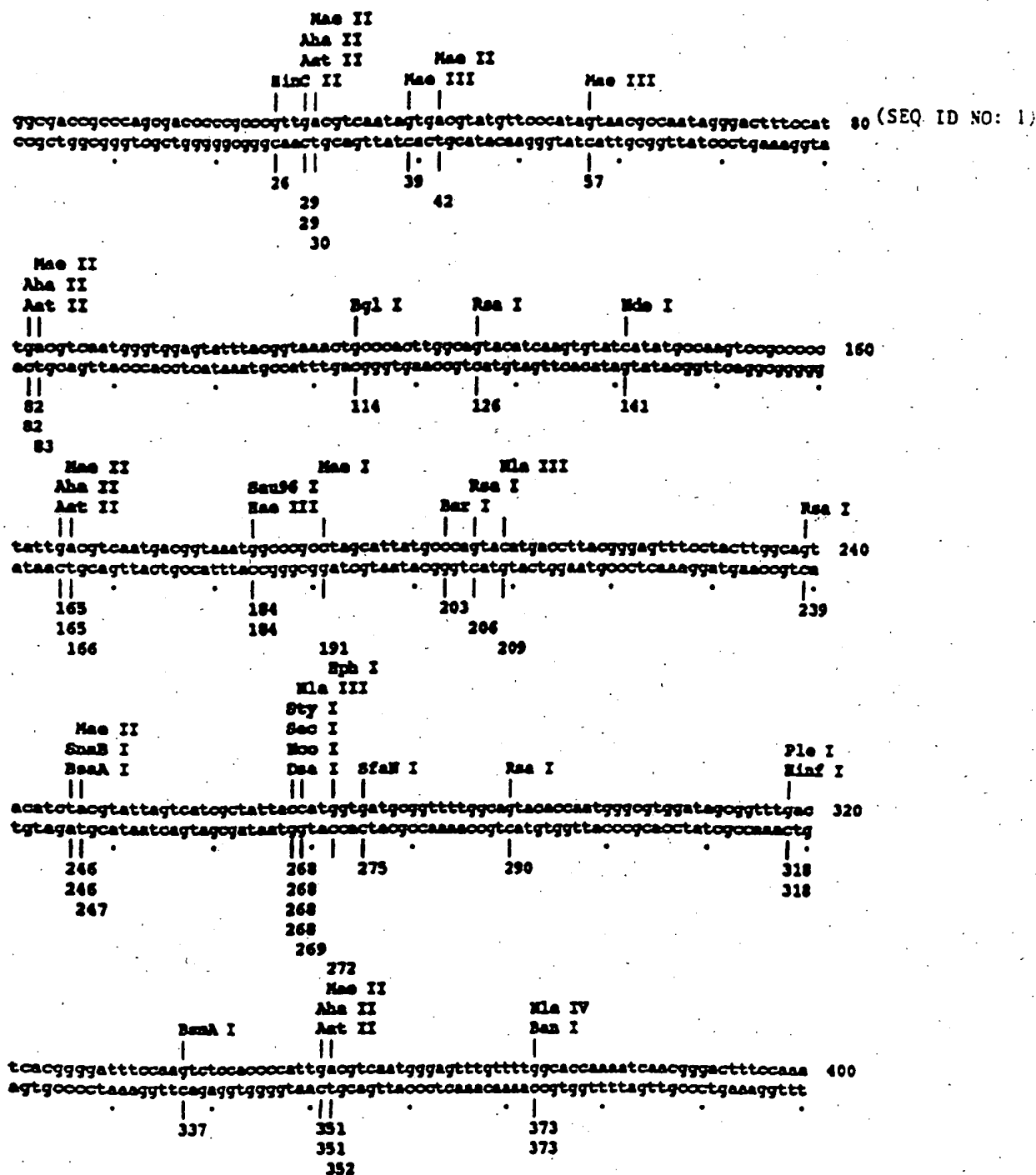
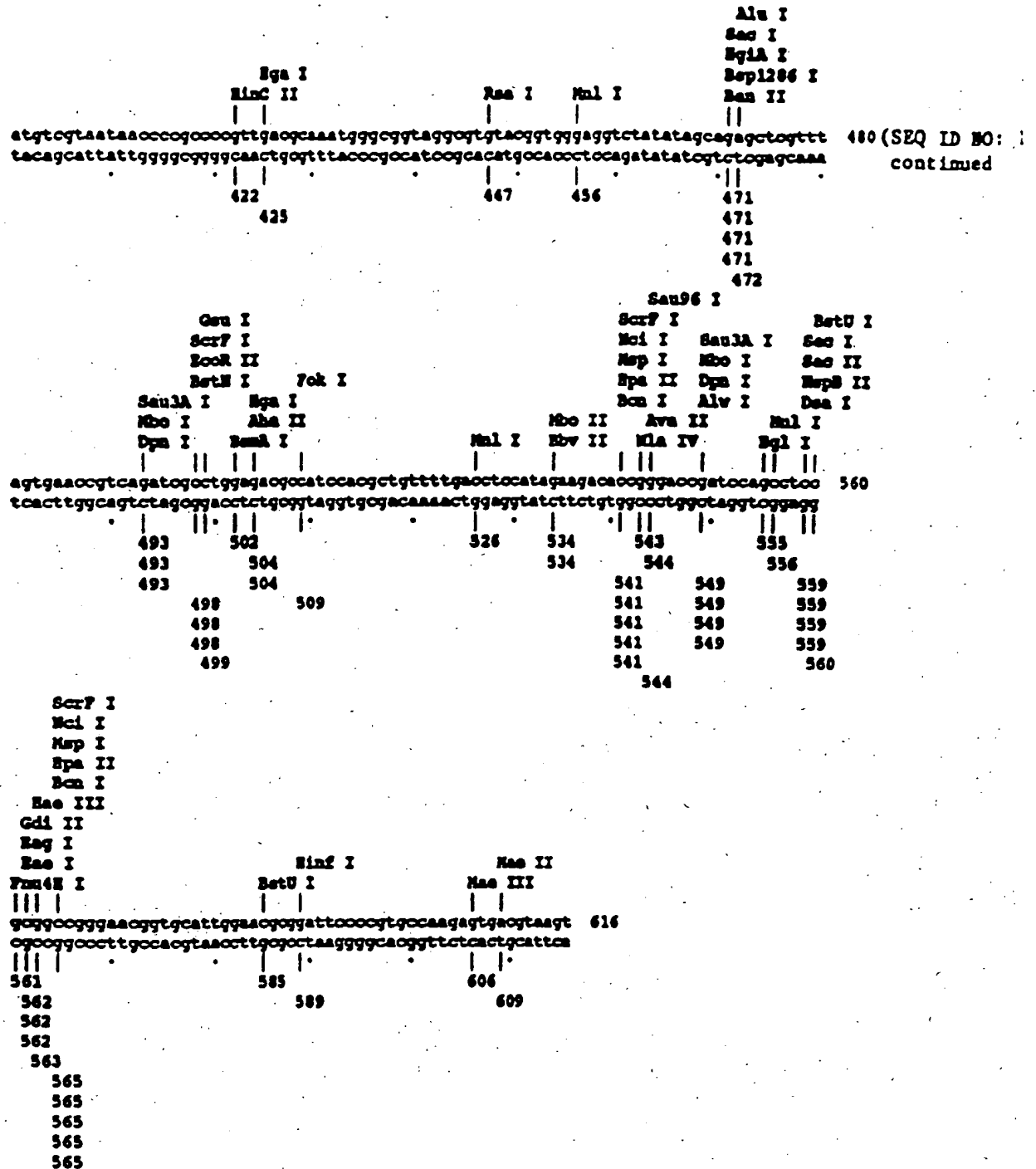


FIGURE 6A(1)

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## Restriction Endonucleases site usage

Aat II	4	BspI I	-	EcoR V	-	Mnl I	3	Rsr II	-
Acc I	-	BspM I	-	Esp I	-	Msc I	-	Sac I	1
Afl II	-	BspM II	-	Fnu4H I	1	Mse I	-	Sac II	1
Afl III	-	Bsr I	1	Fok I	1	Msp I	2	Sal I	-
Aha II	5	BssH II	-	Fep I	-	Mae I	-	Sau3A I	2
Alu I	1	BstB I	-	Gdi II	1	Nar I	-	Sau96 I	2

Alv I	1	BstE II	-	Gen I	1	Hoi I	2	Scn I	-
AlvH I	-	BstH I	1	Kae I	-	Hoo I	1	Scrf I	3
Apa I	-	BstU I	2	Kae II	-	Hda I	1	Sec I	2
Apal I	-	BstI I	-	Kae III	2	Kha I	-	SfaH I	1
Ase I	-	BstY I	-	Sga I	2	Kla III	2	Sfi I	-
Asp718	-	Bsu36 I	-	SgIA I	1	Kla IV	2	Sma I	-
Ava I	-	Cfr10 I	-	SgII II	-	Mot I	-	Snab I	1
Ava II	1	Clu I	-	Kha I	-	Kru I	-	Spe I	-
Avr II	-	Dde I	-	Kinc II	2	Mai I	-	Sph I	-
BamI I	-	Dpn I	2	Kind III	-	Nap7524 I	-	Spl I	-
Ban I	1	Dra I	-	Kinf I	2	Nap8 II	1	Sep I	-
Ban II	1	Dra III	-	Kinf I	-	Nap8 I	-	Sta I	-
Hbe I	-	Drd I	-	Spa I	-	Pac I	-	Sty I	1
Hbv I	-	Dea I	2	Sph II	2	PaeR7 I	-	Taq I	-
Hbv II	1	Eae I	1	Sph I	1	PfIM I	-	TthIII I	-
Hcl I	-	Eag I	1	Kpn I	-	Ple I	1	TthIII II	-
Hcn I	2	Ear I	-	Mae I	1	Pal I	-	Kba I	-
Hgl I	2	Eco47 III	-	Mae II	7	PpuH I	-	Kcm I	-
Hgl II	-	Eco57 I	-	Mae III	3	Pst I	-	Kho I	-
HsaA I	1	EcoN I	-	Mbo I	2	Pvu I	-	Kcm I	-
Hsu I	-	EcoO109 I	-	Mbo II	1	Pvu II	-	Ksa I	-
HsaA I	2	EcoR I	-	Klu I	-	Psa I	5	Kss I	-
Hsp1286 I	1	EcoR II	1	Mne I	-				

Enzyme	Site	Use	Site position (Fragment length) Fragment order			
Alu I	ag/ct	1	1( 471) 1	472( 145) 2		
Alv I	ggato	4/5	1( 548) 1	549( 68) 2		
Ava II	g/gwoc	1	1( 543) 1	544( 73) 2		
Ban I	g/qyroc	1	1( 372) 1	373( 244) 2		
Ban II	grgcy/c	1	1( 470) 1	471( 146) 2		
Hbv II	gaagac	2/6	1( 533) 1	534( 83) 2		
HsaA I	yac/gtr	1	1( 245) 2	246( 371) 1		
Hsp1286 I	gdgch/c	1	1( 470) 1	471( 146) 2		
Bar I	actgg	1/-1	1( 202) 2	203( 414) 1		
BstH I	cc/wgg	1	1( 497) 1	498( 119) 2		
Kae I	y/ggocr	1	1( 561) 1	562( 55) 2		
Eag I	c/ggocg	1	1( 561) 1	562( 55) 2		
EcoR II	/ccwgg	1	1( 497) 1	498( 119) 2		
Fnu4H I	gc/ngc	1	1( 560) 1	561( 56) 2		
Fok I	ggatg	9/13	1( 508) 1	509( 108) 2		
Gdi II	yggocg	-3/-1	1( 561) 1	562( 55) 2		
Gen I	ctggag	16/14	1( 498) 1	499( 118) 2		
SgIA I	gtgcu/c	1	1( 470) 1	471( 146) 2		
Sph I	ggtga	8/7	1( 271) 2	272( 345) 1		
Mae I	c/tag	1	1( 190) 2	191( 426) 1		
Mbo II	gaaga	8/7	1( 533) 1	534( 83) 2		
Hoo I	c/catgg	1	1( 267) 2	268( 349) 1		
Hda I	ca/tatg	1	1( 140) 2	141( 476) 1		
Nap8 II	cmg/ckg	1	1( 558) 1	559( 58) 2		
Ple I	gagtc	4/5	1( 317) 1	318( 299) 2		
Sac I	gagct/c	1	1( 470) 1	471( 146) 2		
Sac II	ccgc/gg	1	1( 558) 1	559( 58) 2		
SfaH I	gcato	5/9	1( 274) 2	275( 342) 1		
Snab I	tac/gta	1	1( 245) 2	246( 371) 1		
Sty I	c/cwgg	1	1( 267) 2	268( 349) 1		
Bcn I	ccs/gg	2	1( 540) 1	541( 24) 3	545( 52) 2	
Bgl I	gcnnnnn/nggc	2	1( 113) 2	114( 441) 1	555( 62) 3	
BamA I	gtata	1/3	1( 336) 1	337( 165) 2	502( 115) 3	
BstU I	cg/cg	2	1( 559) 1	560( 25) 3	585( 32) 2	
Dpn I	ga/to	2	1( 492) 1	493( 56) 3	549( 68) 2	
Dea I	c/crygg	2	1( 267) 2	268( 291) 1	559( 58) 3	
Kae III	gg/cc	2	1( 183) 2	184( 379) 1	563( 54) 3	
Sga I	gaagc	5/10	1( 424) 1	425( 79) 3	504( 113) 2	



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Hinc II	gtg/rac	2	1( 25) 3	26( 396) 1	422( 199) 3	
Hinf I	g/anto	2	1( 317) 1	318( 273) 2	589( 28) 3	
Hpa II	c/ogg	2	1( 540) 1	541( 24) 3	563( 52) 2	
Hbo I	/gate	2	1( 492) 1	493( 56) 3	549( 68) 2	
Hsp I	c/ogg	2	1( 540) 1	541( 24) 3	563( 52) 2	
Hci I	cc/ogg	2	1( 540) 1	541( 24) 3	563( 52) 2	
Hla III	catg/	2	1( 208) 2	209( 60) 3	269( 348) 1	
Hla IV	ggm/noc	2	1( 372) 1	373( 170) 2	543( 74) 3	
SauIA I	/gate	2	1( 492) 1	493( 56) 3	549( 68) 2	
Sau96 I	g/gmcc	2	1( 183) 2	184( 360) 1	544( 73) 3	
Sec I	c/cnogg	2	1( 267) 2	268( 291) 1	559( 58) 3	
Mae III	/gtac	3	1( 38) 2	39( 18) 3	57( 549) 1	606( 11) 4
Mnl I	oote	3	1( 455) 1	456( 70) 2	526( 30) 4	556( 61) 3
ScrF I	cc/ogg	3	1( 497) 1	498( 43) 3	541( 24) 4	565( 52) 2
Ant II	gaagt/c	4	1( 28) 5 351( 266) 1	29( 53) 4	82( 83) 3	165( 186) 2
Aha II	gr/oggo	5	1( 28) 6 351( 153) 2	29( 53) 3 504( 113) 3	82( 83) 4	165( 186) 1
Rsa I	gt/ac	5	1( 125) 3 290( 157) 2	126( 80) 4 447( 170) 1	206( 33) 6	239( 51) 5
Mae II	a/cgt	7	1( 29) 6 166( 81) 4	30( 12) 7 247( 105) 2	42( 41) 5 352( 257) 1	83( 83) 3 609( 8) 8

98 sites found

No Sites found for the following Restriction Endonucleases

Aac I	gt/mkac		Dra III	cacnna/gtg	Hsp7524 I	r/catgy
Afl II	c/ttaag		Drd I	gacnna/ngtc	HspI I	roatg/y
Afl III	a/crygt		Ear I	ctcttc 1/4	Pae I	ttaat/taa
AlwI I	cagnnn/ctg		Eco47 III	ago/gct	PaeR7 I	c/togag
Apa I	gggoc/c		Eco57 I	ctgaag 16/14	PflM I	ccannnn/ntgg
ApaL I	g/tgcac		EcoN I	ccnna/nnnag	Pal I	cac/gtg
Ase I	at/taat		EcoO109 I	rg/gmcc	PpmI I	rg/gmcc
Asp718	g/gtacc		EcoR I	g/anttc	Pst I	ctga/g
Ava I	c/yogrg		EcoR V	gat/ato	Pvu I	oggt/og
Avr II	c/ctagg		Esp I	gc/taagc	Pvu II	cag/ctg
BamI I	g/gatcc		Fsp I	tgc/gca	Rsr II	og/gmcc
Ebe I	gggoc/c		Eae I	vvg/ocw	Sal I	g/togac
Ebv I	gcagc	8/12	Eae II	rgcga/y	Sca I	agt/aot
Ecl I	t/gatca		EglII II	acnnnnnnnggt	Sfi I	ggcnnnnn/nggoc
Egl II	a/gatct		Eha I	ggg/c	Sma I	ccc/ggg
Bem I	gaatgc	1/-1	Hind III	a/agctt	Spe I	a/ctagt
BspI I	t/catga		HinP I	g/ogo	Sph I	gcatt/g
BspH I	acatgc	4/8	Hpa I	gtt/aac	Spl I	c/gtaag
BspH II	t/ocgga		Hpa I	ggtac/c	Ssp I	aat/att
BssII II	g/ogogo		Hla I	a/ogogt	Sta I	agg/cot
BstI I	tt/cgaa		Hma I	tcacac 20/18	Taq I	t/cga
BstE II	g/gtnacc		Hsc I	tgg/oca	TthIII I	gacn/ngtc
BstI I	ccannnnn/ntgg		Hse I	t/taa	TthIII II	caarca 11/9
BstY I	r/gatcy		Hae I	goc/ggc	Tba I	t/ctaga
Bsu36 I	cc/taagg		Har I	gg/ogcc	Tca I	gta/tac
Cfr10 I	cc/oggy		Hhe I	g/ctaga	Tco I	c/togag
Cla I	at/cgat		Hol I	gc/ggocgc	Tcm I	ccannnnn/annntgg
Dde I	c/tnag		Hru I	toq/cga	Tma I	c/ocggg
Dra I	ttt/aaa		Hsi I	atgca/t	Tmi I	gaann/nnttc

FIGURE 6A(4)

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\*\*\* Aligned sequences:

C1 ( 1f): |>u 1>++++ ad169hcmv (930 bases)++++>u 930>  
 C2 ( 1f): |>u 1>++++ townehcmv (616 bases)++++>u 616>

\*\*\* Alignment of first sequence with all others displayed

\*\*\* Key:

UPPER CASE = aligned non-identical bases

lower case = unaligned bases

----- = aligned identical bases

..... = gap

SEQ ID

NO: 2)

SEQ ID

NO: 3)

```

ad169hcmv : AATCAATATTGGCCATTAGCCATATTATTCATTGGTTATATAGCATAAATCAATATTGGC
townehcmv : .....

ad169hcmv : TATTGGCCATTGCATACGTTGTATCCATATCATAATATGTACATTATATTGGCTCATGT
townehcmv : .....

ad169hcmv : CCAACATTACCGCCATGTTGACATTGATTATTGACTAGTTATTAATAGTAATCAATTACG
townehcmv : .....

ad169hcmv : GGGTCATTAGTTCATAGCCCATATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGC
townehcmv : .....

ad169hcmv : CCGCCTGGCTGACCGCCCAACGACCCCGCCCATTTGACGTCATAATGACGTATGTTCCC
townehcmv : .....-G-----G-----G-----

ad169hcmv : ATAGTAACGCCAATAGGGACTTTCCATTGACGTCATGGGTGGAGTATTTACGGTAAACT
townehcmv : -----

ad169hcmv : GCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAT
townehcmv : -----C-----

ad169hcmv : GACGGTAAATGGCCCCCTGGCATTATGCCCAAGTACATGACCTTATGGGACTTTCTACT
townehcmv : -----A-----C-----G-----

ad169hcmv : TGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATCGGTTTTGGCAGTAC
townehcmv : -----

ad169hcmv : ATCAATGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTTGAC
townehcmv : -C-----

ad169hcmv : GTCAATGGGAGTTTGTTTTGGCACCAAAATCAACGGGACTTTCCAAATGTCGTAAACAAC
townehcmv : -----T-----

ad169hcmv : TCCGCCCCATTGACGCAAAATGGCGGTAGGCGGTACGGTGGGAGGTCTATATAAGCAGA
townehcmv : C-----G-----

ad169hcmv : GCTCGTTTAGTGAACCGTCAGATCGCCTGGAGACGCCATCCACGCTGTTTGACCTCCAT
townehcmv : -----

ad169hcmv : AGAAGACACCGGGACCGATCCAGCCTCCGCGGCCGGGAACGGTGCAATGGAAACGCGGATT
townehcmv : -----

ad169hcmv : CCCCCTGCCAAGAGTGACGTAAGTACCGCCTATAGAGTCTATAGCCCCCCCCCTTGCT
townehcmv : -----

ad169hcmv : TCTTATGCATGCTATACTGTTTTTGGCTTG
townehcmv : .....

```

FIGURE 6B(1).

SUBSTITUTE SHEET

12/45

LOCUS BS51EE 930 bp ds-DNA VRL 15-SEP-1989  
 DEFINITION Human cytomegalovirus major immediate-early gene, enhancer.  
 ACCESSION K03104  
 KEYWORDS major immediate-early gene.  
 SOURCE HCMV strain AD169.  
 ORGANISM Human cytomegalovirus  
 Viridae; ds-DNA enveloped viruses; Herpesviridae;  
 Betaherpesvirinae.  
 REFERENCE 1 (bases 1 to 930)  
 AUTHORS Boshart, M., Weber, F., Jahn, G., Dorsch-Baesler, K., Fleckenstein, B.  
 and Schaffner, W.  
 TITLE A very strong enhancer is located upstream of an immediate early  
 gene of human cytomegalovirus  
 JOURNAL Cell 41, 521-530 (1985)  
 STANDARD full automatic  
 REFERENCE 2 (sites)  
 AUTHORS Zhang, X.-Y., Inandur, N.M., Supakar, P.C., Wu, X., Ehrlich, M. and  
 Ehrlich, K.C.  
 TITLE three MDSBP sites in the immediate-early enhancer-promoter region of  
 human cytomegalovirus  
 JOURNAL Virology 182, 865-869 (1991)  
 STANDARD full automatic  
 COMMENT Draft entry and printed copy of sequence in [1] were kindly  
 provided by M.Boshart, 24-OCT-1985.

The sequence shown is a 930 bp segment of the PstI m-fragment from  
 HCMV strain AD169. The enhancer region of the HCMV gene was defined  
 by selecting for fragments of HCMV DNA that would restore efficient  
 growth of enhancerless SV40.

A possible splice donor signal is located at positions 856-864.  
 FEATURES Location/Qualifiers  
 misc\_signal 214..620  
 /note="HCMV IE enhancer region"  
 mRNA 738..>930  
 /note="HCMV IE mRNA"  
 (SEQ ID NO: 2)  
 BASE COUNT 233 a 228 c 211 g 258 t  
 ORIGIN 12 bp upstream of Bali site; .750 mu.

```

1 aatcaatatt ggccattagc catattattc attgggtata tagcataaat caatattggc
61 tattggccat tgcatacytt gtatccatat cataatatgt acatttatat tggctcatgt
121 ccaacattac cgccatgttg acattgatta ttgactagtt attaatagta atcaattacg
181 gggtcattag ttcatagcc atatatggag ttccgcgta cataacttac ggtaaatggc
241 ccgcctggct gaccgcccaa cgacccccgc coattgacgt caataatgac gtatgttccc
301 atagtaacgc caatagggac ttccattga cgtcaatggg tggagtattt acggtaaac
361 gccacttg cagtacatca agtgatcat atgccaagta cgcacctat tgacgtcaat
421 gacggtaaat ggccgcctg gcattatgcc cagtacatga cttatggga ctttctact
481 tggcagtaca tctacgtatt agtcacgct attaccatgg tgatgcggtt ttggcagtac
541 atcaatgggc gtggatagcg gtttgactca cggggatttc caagtctcca cccattgac
601 gtcaatggga gtttgttttg gcacccaaat caacgggact ttccaaatg tcgtaacaac
661 tccgccccat tgacgcaaat gggcggtagg cgtgtacggt gggaggtcta tataagcaga
721 gctcggttag tgaaccgtca gatcgcttgg agacgccatc cacgctgtt tgacctccat
781 agaagacacc gggacggatc cagcctccgc ggcgggaac ggtgcattgg aacgcggatt
841 cccgtgcc aagtgacgt aagtaaccgc tatagagtct ataggccac ccccttggt
901 tcttatgcat gotatactgt ttttggtt
```

FIGURE 6B(2)

SUBSTITUTE SHEET

13/45

LOCUS BS5MIE1 616 bp ds-DNA VRL 15-SZP-1989  
 DEFINITION Human cytomegalovirus (Towne) major immediate-early (IE) gene, exon 1.  
 ACCESSION K01484 K01090  
 KEYWORDS major immediate-early gene.  
 SEGMENT 1 of 4  
 SOURCE Human cytomegalovirus (strain Towne) passed in primary human foreskin fibroblasts, DNA [1], clone pXEP22 [2].  
 ORGANISM Human cytomegalovirus  
 Viridae; ds-DNA enveloped viruses; Herpesviridae; Betaherpesvirinae.  
 REFERENCE 1 (bases 460 to 616)  
 AUTHORS Stenberg, R.M., Thomsen, D.R. and Stinski, M.F.  
 TITLE Structural analysis of the major immediate early gene of human cytomegalovirus  
 JOURNAL J. Virol. 49, 190-199 (1984)  
 STANDARD full automatic  
 REFERENCE 2 (bases 1 to 490)  
 AUTHORS Thomsen, D.R., Stenberg, R.M., Goins, W.F. and Stinski, M.F.  
 TITLE Promoter-regulatory region of the major immediate early gene of human cytomegalovirus  
 JOURNAL Proc. Natl. Acad. Sci. U.S.A. 81, 659-663 (1984)  
 STANDARD full automatic  
 COMMENT IE region 1 gene is also known as the major IE gene.

Cytomegalovirus immediate-early gene expression is dominated in vivo by the expression of a single gene. At least three promoters influence transcription of the virus after infection. When a complete set of promoter-regulatory regions were present (IE regions 1, 2 and 3 or IE region 1 and an adenovirus major late promoter), transcription was qualitatively higher from IE region 1.

Based on these data, [1] proposes that the upstream sequence of the IE region 1 gene competes more efficiently for RNA polymerase II or other host cell proteins necessary for in vitro transcription.

Consensus CAAT and TATA boxes were found at positions 429-433 and 462-467, a polyadenylation signal was found at positions 2198-2203.

Fourteen direct repeats were found in the promoter-regulatory region (four 16-bp repeats, four 18-bp repeats, four 19-bp repeats and two 21-bp repeats).

Draft entry and clean copy sequences [1], [2] kindly provided by P.R. Witte and M.F. Stinski (10-FEB-1986).

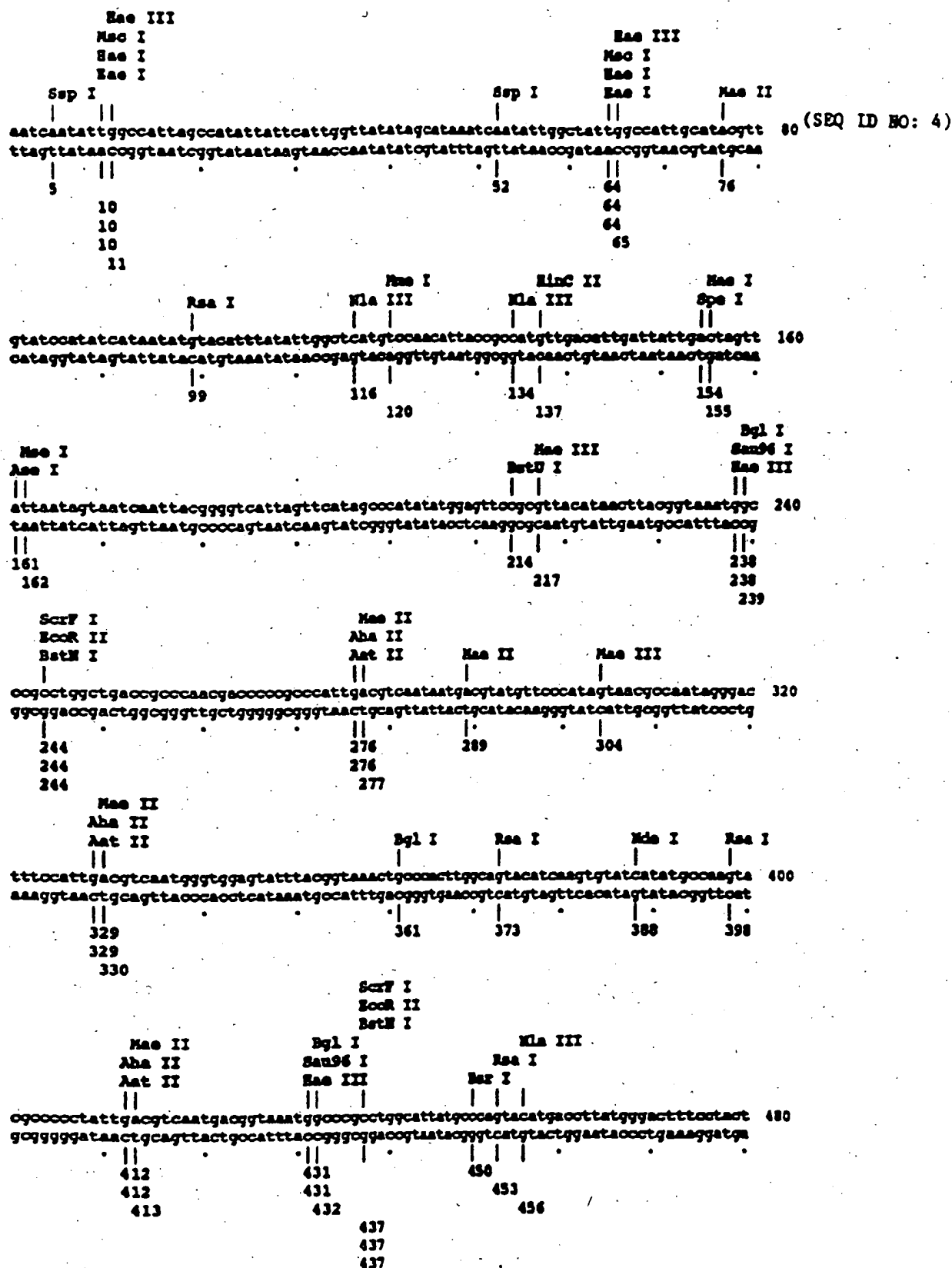
FEATURES Location/Qualifiers  
 prim\_transcript 490..>616  
 /note="major IE mRNA"  
 intron 611..>616  
 /note="major IE mRNA intron A" (SEQ ID NO: 3)  
 BASE COUNT 144 a 165 c 162 g 145 t  
 ORIGIN 28 bp upstream of BincII site; 0.752 map units.  
 1 ggcgaccgcc cagcgacccc cgcgcgttga cgtcaatagt gacgtatgtt cccatagtaa  
 61 cgccaatagg gactttccat tgacgtcaat ggggtggagta ttacggtaa actgccact  
 121 tggcagtaca tcaagtgtat catatgccaa gtccgcccc tattgacgtc atgacggta  
 181 aatggcccg ctagcattat gccagtagca tgacctacg ggagtttcc acttggcagt  
 241 acatctacgt attagtcac gctattacca tgggtgatgc gttttggcag tacaccaatg  
 301 ggcgtggata gcggtttgac tcacggggat ttccaagtct ccacccatt gacgtcaatg  
 361 ggagtttgtt ttggcaccas aatcaacggg actttccaaa atgtcgtaat aaccccgccc  
 421 cgttgacgca aatggcggt aggcgtgtac ggtgggaggt ctatatagca gacgtcgtt  
 481 agtgaaccgt cagatcgcc tggagacgcca tccacgtgt tttgacctcc atagaagaca  
 541 ccgggaaccga tccagcctcc gcggccggga acggtgcatt ggaacgcgga ttcccctgc  
 601 caagagtgc gtaagt

SUBSTITUTE SHEET

FIGURE 08(3)

14/45

Positions of Restriction Endonucleases sites (unique sites underlined)



(SEQ ID NO: 4)

continued

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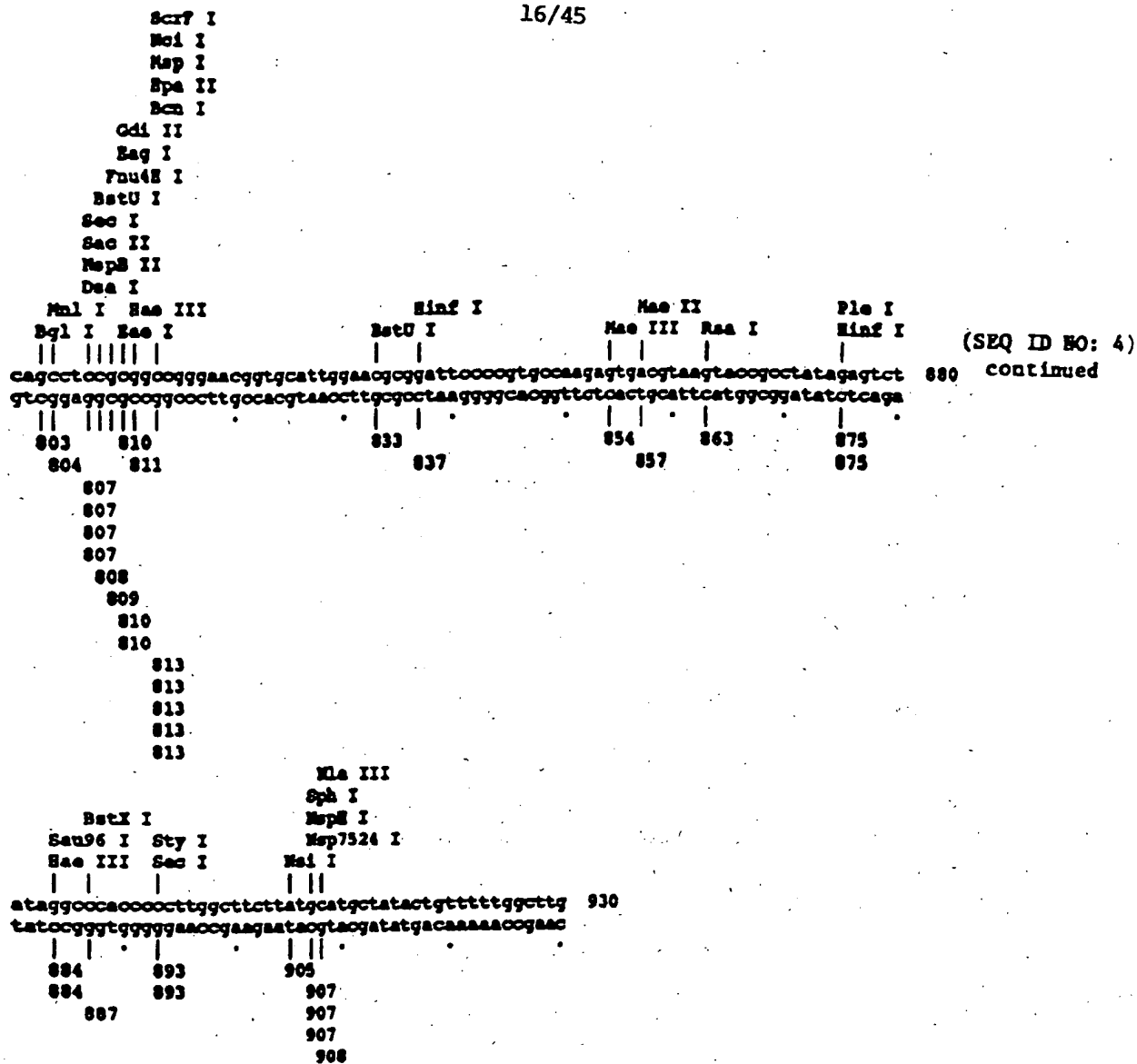
2870

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**FIGURE 6C(2)**

# SUBSTITUTE SHEET

16/45



## Restriction Endonucleases site usage

Aat II	4	BspI I	-	EcoR V	-	Mnl I	3	Nar II	-
Acc I	-	BspM I	-	Esp I	-	Nco I	2	Sac I	1
Afl II	-	BspM II	-	Fnu4H I	1	Nde I	1	Sac II	1
Afl III	-	Bar I	1	Fok I	1	Nsp I	2	Sal I	-
Aha II	5	Ban II	-	Fsp I	-	Nse I	-	SauIA I	2
Alu I	1	BstB I	-	Gdi II	1	Nar I	-	Sau96 I	4
Alw I	1	BstE II	-	Gsu I	1	Nci I	2	Sca I	-
AlwI I	-	BstH I	3	Hae I	2	Nco I	1	SclI I	5
Apa I	-	BstU I	3	Hae II	-	Nde I	1	Sac I	3
Apal I	-	BstX I	1	Hae III	6	Nhe I	-	SfaI I	1
Ase I	1	BstY I	-	Hpa I	2	Nla III	5	Sfi I	-
Asp718	-	Bru36 I	-	HgiA I	1	Nla IV	2	Sna I	-
Ava I	-	Cfr10 I	-	HgiE II	-	Not I	-	SnaB I	1
Ava II	1	Cla I	-	Hha I	-	Nru I	-	Spe I	1
Avr II	-	Dde I	-	Hinc II	1	Nsi I	1	Sph I	1
BamI I	-	Dpn I	2	Hind III	-	Nsp7524 I	1	Spl I	-
Ban I	1	Dra I	-	Hinf I	3	NspB II	1	Ssp I	2
Ban II	1	-	-	Hle I	-	NspI I	1	Stu I	-

Bbe I	-	Drd I	-	Spa I	-	Pac I	-	Sty I	2
Bbv I	-	Dea I	2	Spa II	2	PaeR7 I	-	Taq I	-
Bbv II	1	Eae I	3	Sph I	1	PflM I	-	TthIII I	-
Bcl I	-	Eag I	1	Xpn I	-	Ple I	2	TthIII II	-
Bcn I	2	Ear I	-	Mae I	1	Pal I	-	Xba I	-
Bgl I	4	Eco47 III	-	Mae II	8	PpuM I	-	Xca I	-
Bgl II	-	Eco57 I	-	Mae III	4	Pst I	-	Xho I	-
BsaA I	1	EcoM I	-	Kho I	2	Pvu I	-	Xcm I	-
Bsa I	-	EcoO109 I	-	Kho II	1	Pvu II	-	Xma I	-
BsaA I	2	EcoR I	-	Mla I	-	Rsa I	8	Xmn I	-
Bsp1286 I	1	EcoR II	3	Mms I	1				

Enzyme	Site	Use	Site position (Fragment length)		Fragment order	
Alu I	ag/ct	1	1( 719)	1	720( 211)	2
Alv I	ggato	4/5	1( 796)	1	797( 134)	2
Ase I	at/tant	1	1( 160)	2	161( 770)	1
Ava II	q/gwco	1	1( 791)	1	792( 139)	2
Ban I	q/gyroo	1	1( 619)	1	620( 311)	2
Ban II	grgoy/c	1	1( 718)	1	719( 212)	2
Bbv II	gaagac	2/6	1( 781)	1	782( 149)	2
BsaA I	yac/gtr	1	1( 492)	1	493( 438)	2
Bsp1286 I	gdgch/c	1	1( 718)	1	719( 212)	2
Ber I	actgg	1/-1	1( 449)	2	450( 481)	1
BstI I	ccannnnn/ntgg	1	1( 886)	1	887( 44)	2
Eag I	c/ggcoo	1	1( 809)	1	810( 121)	2
Fnu4H I	gc/ngo	1	1( 808)	1	809( 122)	2
Fok I	ggatg	9/13	1( 756)	1	757( 174)	2
Gdi II	yggoog	-5/-1	1( 809)	1	810( 121)	2
Gru I	ctggag	16/14	1( 746)	1	747( 184)	2
HgiA I	gwgw/c	1	1( 718)	1	719( 212)	2
Hinc II	gty/rac	1	1( 136)	2	137( 794)	1
Hph I	ggtga	8/7	1( 518)	1	519( 412)	2
Hae I	c/tag	1	1( 154)	2	155( 776)	1
Kho II	gaaga	8/7	1( 781)	1	782( 149)	2
Hae I	tcocrac	20/18	1( 119)	2	120( 811)	1
Hae I	t/taa	1	1( 161)	2	162( 769)	1
Hoo I	c/catgg	1	1( 514)	1	515( 416)	2
Hde I	ca/tatg	1	1( 387)	2	388( 543)	1
Hai I	atgaa/t	1	1( 904)	1	905( 26)	2
Hsp7524 I	r/catgg	1	1( 906)	1	907( 24)	2
HspB II	cmg/chg	1	1( 806)	1	807( 124)	2
HspK I	rcatg/y	1	1( 906)	1	907( 24)	2
Sac I	gagct/c	1	1( 718)	1	719( 212)	2
Sac II	ccgc/gg	1	1( 806)	1	807( 124)	2
SfaH I	gcato	5/9	1( 521)	1	522( 409)	2
SnaB I	tac/gta	1	1( 492)	1	493( 438)	2
Spe I	a/ctaqt	1	1( 153)	2	154( 777)	1
Sph I	gcata/c	1	1( 906)	1	907( 24)	2
Bcn I	cos/gg	2	1( 788)	1	789( 24)	3
BsaA I	gtctc	1/5	1( 583)	1	584( 166)	3
Dpn I	ga/ta	2	1( 740)	1	741( 56)	3
Dea I	c/crygg	2	1( 514)	1	515( 292)	2
Hae I	wgg/ccw	2	1( 9)	3	10( 54)	2
Hga I	gaogo	5/10	1( 671)	1	672( 80)	3
Hpa II	c/ogg	2	1( 788)	1	789( 24)	3
Kho I	/gata	2	1( 740)	1	741( 56)	3
Hae I	tgg/cca	2	1( 9)	3	10( 54)	2
Hsp I	c/ogg	2	1( 788)	1	789( 24)	3
Hci I	cc/agg	2	1( 788)	1	789( 24)	3
Hla IV	ggg/noc	2	1( 619)	1	620( 171)	2
Ple I	gagta	4/5	1( 564)	1	565( 310)	2
SauIA I	/gata	2	1( 740)	1	741( 56)	3
Ssp I	aat/att	2	1( 4)	3	5( 47)	2
Sty I	c/cwgg	2	1( 514)	1	515( 378)	2
					893( 38)	3



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BstM I	cc/wgg	3	1( 243) 2	244( 193) 3	437( 309) 1	746( 185) 4
BstU I	cg/cg	3	1( 213) 2	214( 594) 1	808( 25) 4	833( 98) 3
Eae I	y/ggocr	3	1( 9) 4	10( 54) 3	64( 746) 1	810( 121) 2
EcoR II	/ocwgg	3	1( 243) 2	244( 193) 3	437( 309) 1	746( 185) 4
Einf I	g/antc	3	1( 564) 1	565( 272) 2	837( 38) 4	875( 56) 3
Hnl I	ccta	3	1( 702) 1	703( 71) 3	774( 30) 4	804( 127) 2
Sec I	c/cnnqg	3	1( 514) 1	515( 292) 2	807( 86) 3	893( 38) 4
Lat II	gacgt/c	4	1( 275) 2	276( 53) 5	329( 83) 4	412( 186) 3
			598( 333) 1			
Bgl I	gocnnnnn/nggc	4	1( 238) 2	239( 122) 4	361( 71) 5	432( 371) 1
			803( 128) 3			
Mae III	/gtnac	4	1( 216) 2	217( 87) 4	304( 349) 1	653( 201) 3
			854( 77) 5			
Sau96 I	g/gncc	4	1( 237) 2	238( 193) 3	431( 361) 1	792( 92) 4
			884( 47) 5			
Aha II	gr/cgyc	5	1( 275) 1	276( 53) 6	329( 83) 5	412( 186) 2
			598( 154) 4	752( 179) 3		
Kla III	catg/	5	1( 115) 3	116( 18) 6	134( 322) 2	456( 60) 4
			516( 392) 1	908( 23) 5		
SorF I	cc/ngg	5	1( 243) 2	244( 193) 3	437( 309) 1	746( 43) 5
			789( 24) 6	813( 118) 4		
Eae III	gg/cc	6	1( 10) 7	11( 54) 5	65( 173) 3	238( 193) 2
			431( 380) 1	811( 73) 4	884( 47) 6	
Mae II	a/cgt	8	1( 75) 6	76( 201) 2	277( 12) 9	289( 41) 8
			330( 83) 4	413( 81) 5	494( 105) 3	599( 258) 1
			857( 74) 7			
Rsa I	gt/ac	8	1( 98) 4	99( 274) 1	373( 25) 9	398( 55) 6
			453( 33) 8	486( 51) 7	537( 157) 3	694( 169) 2
			863( 68) 5			

141 sites found

No Sites found for the following Restriction Endonucleases

Acc I	gt/mkac		Dde I	c/tnag	Hru I	tcg/cga
Afl II	c/ttaag		Dra I	ttt/aaa	Pae I	ttaat/taa
Afl III	a/crygt		Dra III	caacnnn/gtg	Paar7 I	a/togag
AluI	caacnnn/ctg		Erd I	gacnnnn/ngtgc	PfII I	ccannnn/ntgg
Apa I	gggoc/c		Esr I	ctcttc 1/4	Pml I	caa/gtg
ApaL I	g/tgcac		Eco47 III	agc/gct	PpuII I	rg/gwooy
Asp718	g/gtacc		Eco57 I	ctgaag 16/14	Pst I	ctgca/g
Ava I	c/yogrg		EcoRI	ccnnn/bnnagg	Pvu I	cgat/cg
Avr II	c/ctagg		EcoRI09 I	rg/gnocy	Pvu II	caq/ctg
BamI	g/gatcc		EcoRI	g/aatto	Rar II	cg/gwoog
Ebe I	ggogc/c		EcoRV	gat/ato	Sal I	g/togac
Ebv I	gcage	8/12	Esp I	gc/tnagc	Sca I	agt/act
Ecl I	t/gatca		Fsp I	tgc/gca	Sfi I	ggcnnnn/nggoc
Bgl II	a/gatct		Eae II	rgogc/y	Sma I	ccc/ggg
Bam I	gaatgc	1/-1	BglR II	acnnnnnnnggt	Spl I	c/gtaog
BspI	t/catga		Eba I	gog/c	Stu I	agg/oct
BspH I	acctgc	4/8	KlnB III	a/agctt	Taq I	t/cga
BspH II	t/cooga		KlnP I	g/ogo	TthIII I	gacn/ngtgc
BesH II	g/ogoc		Spa I	ggt/aaa	TthIII II	caarea 11/9
BstI	tt/cgaa		Xpn I	ggtac/c	Xba I	t/ctaga
BstE II	g/gtnaoc		Mlu I	a/ogogt	Xba I	gta/tac
BstY I	r/gatoy		Mae I	goc/ggc	Xho I	c/togag
Bsu36 I	cc/tnagg		Nar I	gg/ogoo	Xcm I	ccannnnn/nnntgg
Cfr10 I	r/coogy		Nbe I	g/ctagc	Xma I	c/coogy
Cla I	at/ogat		Nci I	gc/ggocgc	Xmn I	gaann/nnttc

## INTERNALIZATION OF LIPID DNA COMPLEXES IN CV-1 CELLS

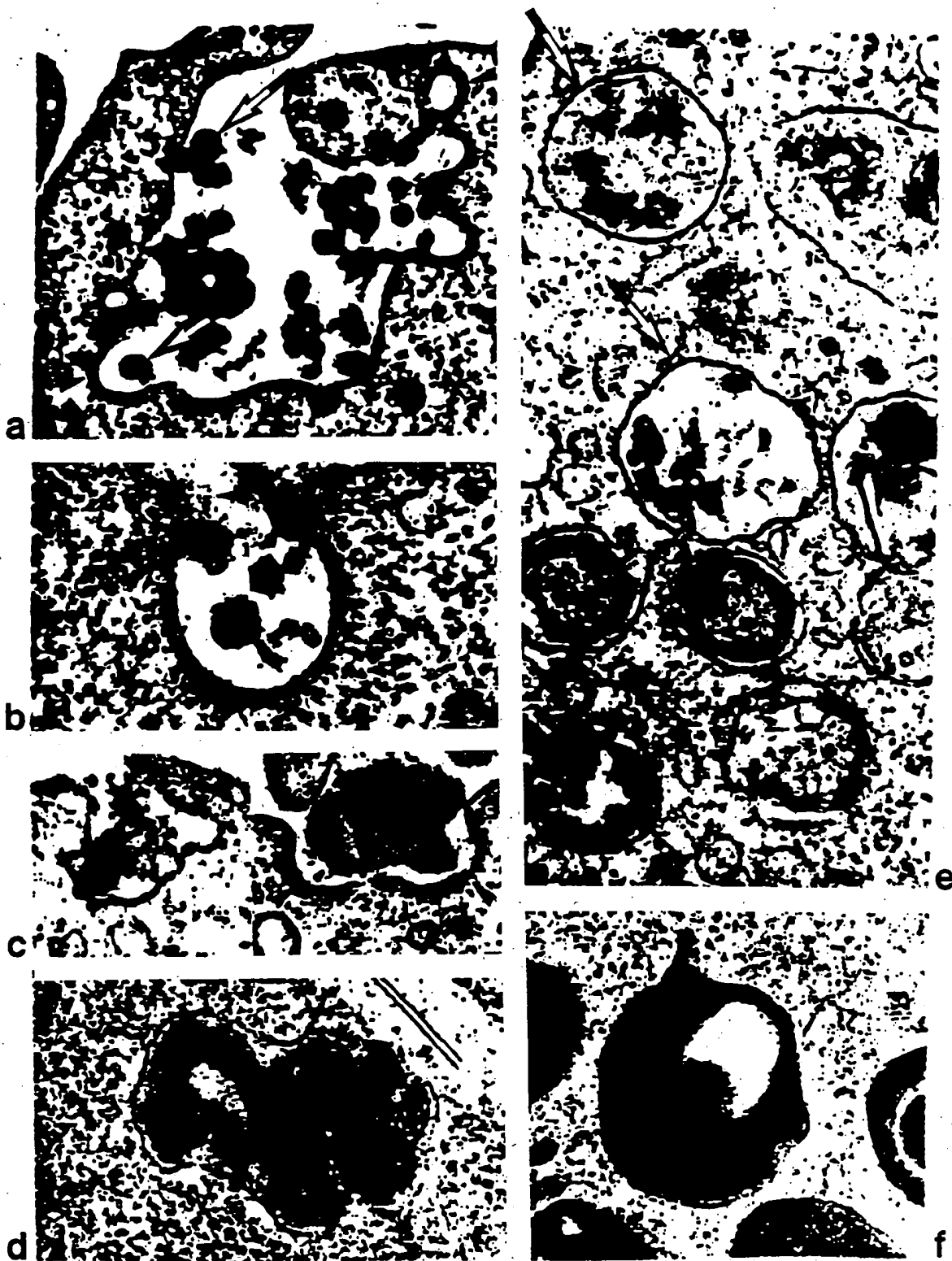


FIGURE 7  
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FIGURE 8A



FIGURE 8B

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FIGURE 8C

Control

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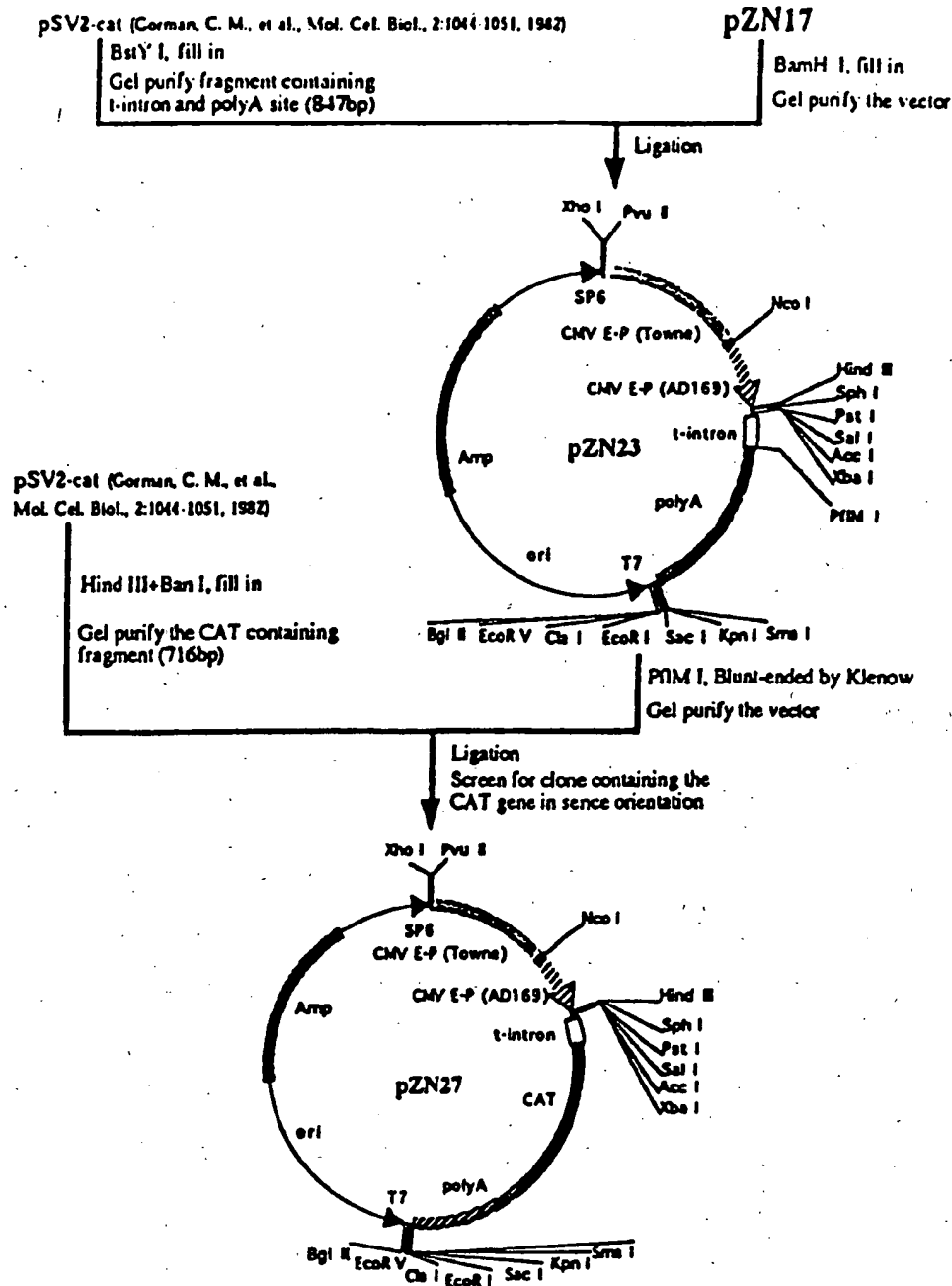
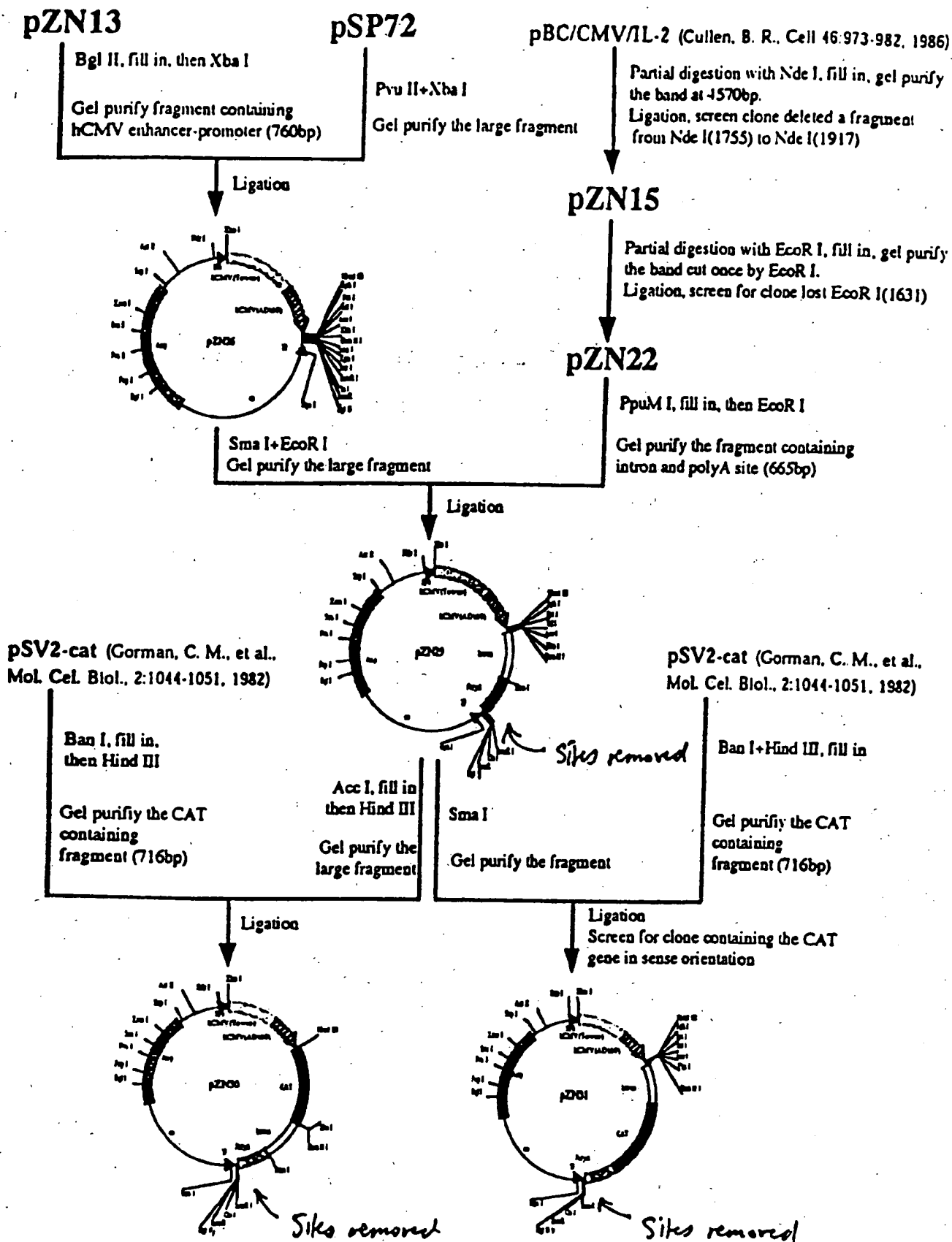


Figure 9  
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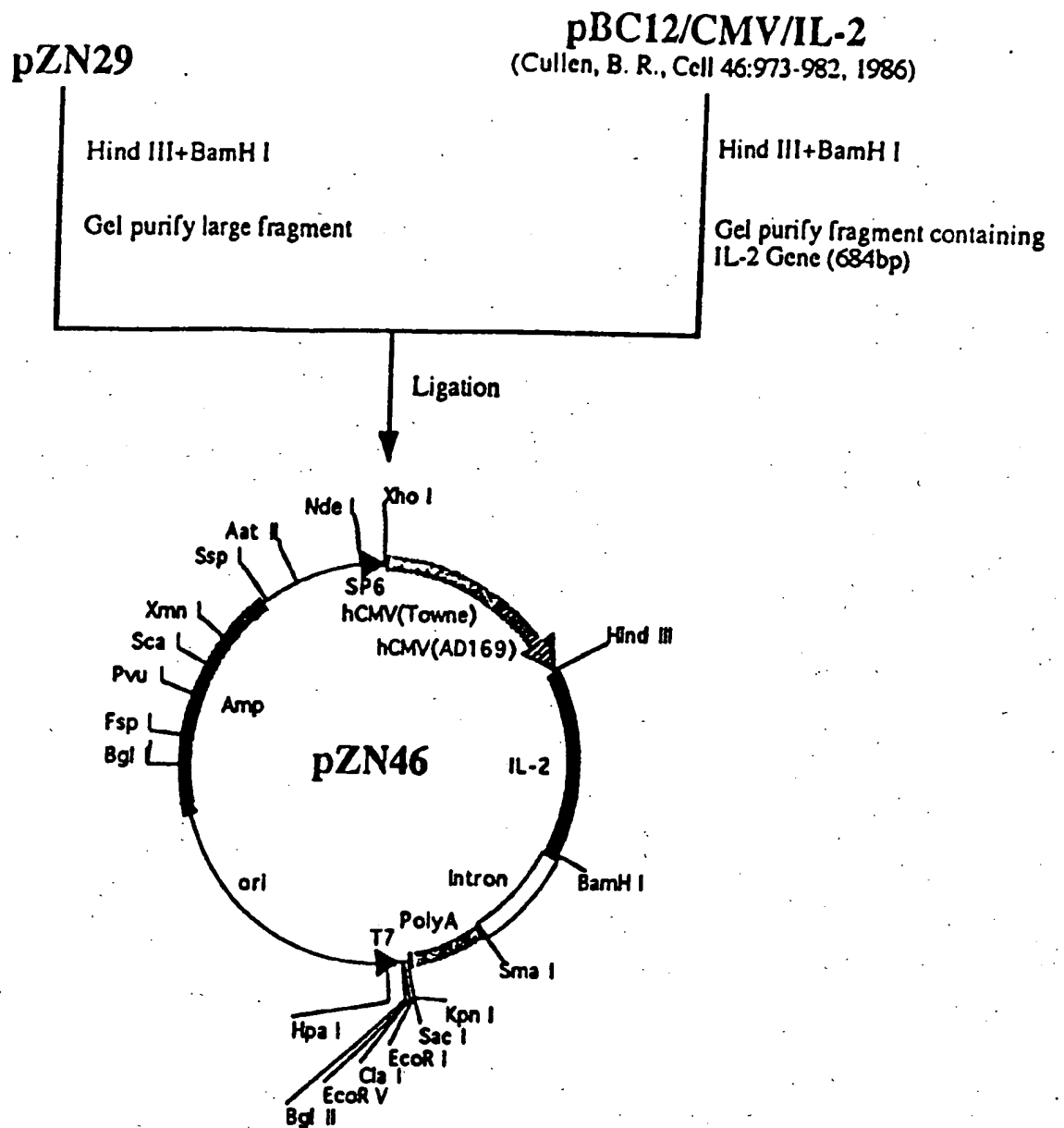


Figure 10B  
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pBQ4.7 (CFTR cDNA clone, from Dr. Francis Collins)

pZN29

Pst I, fill in, gel purify CFTR cDNA Insert (~4.7kb)

Sma I

Ligation

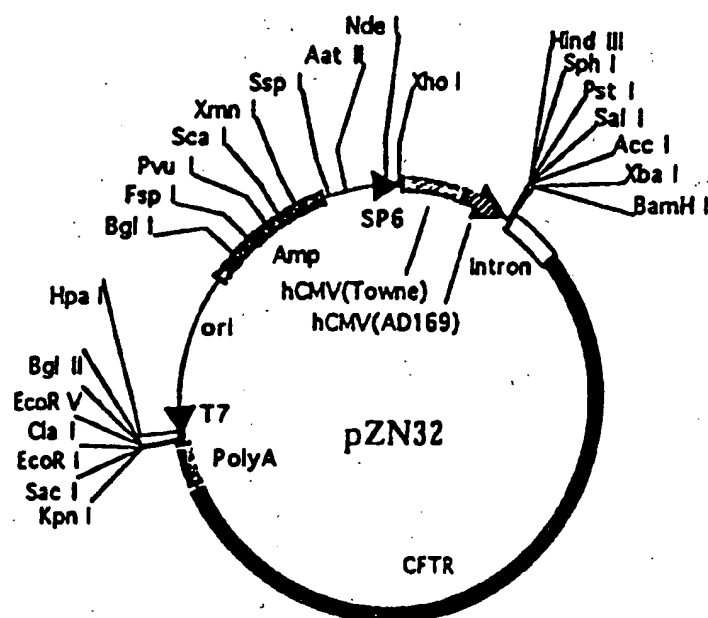
Screen for clone containing insert  
in the sense orientation

Figure 11  
SUBSTITUTE SHEET



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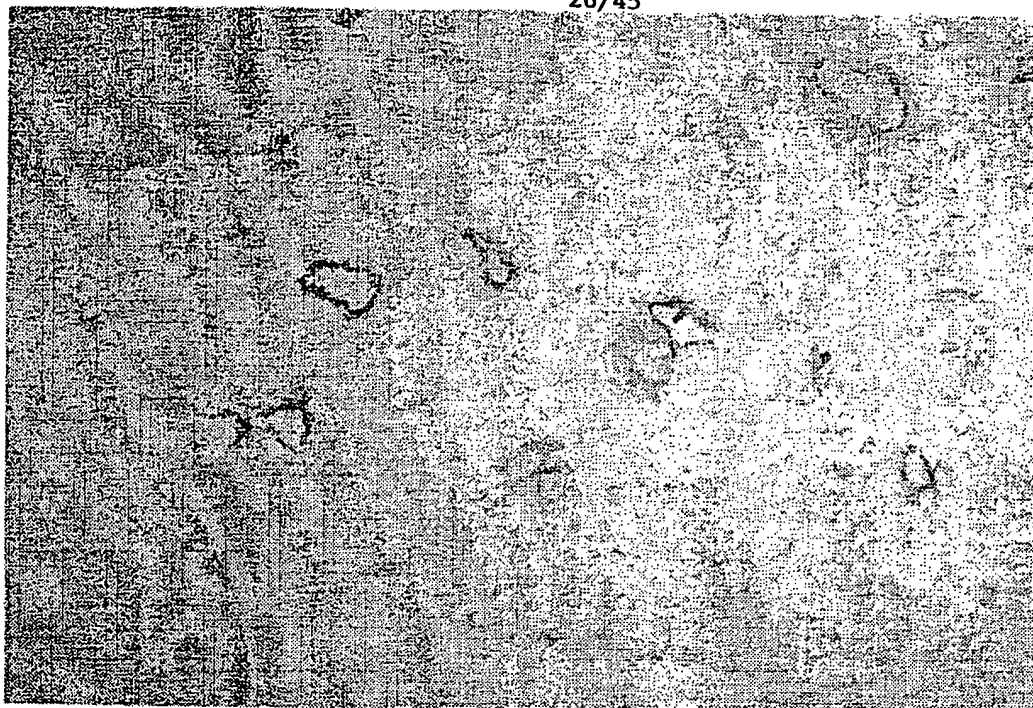


FIGURE 12A

50X

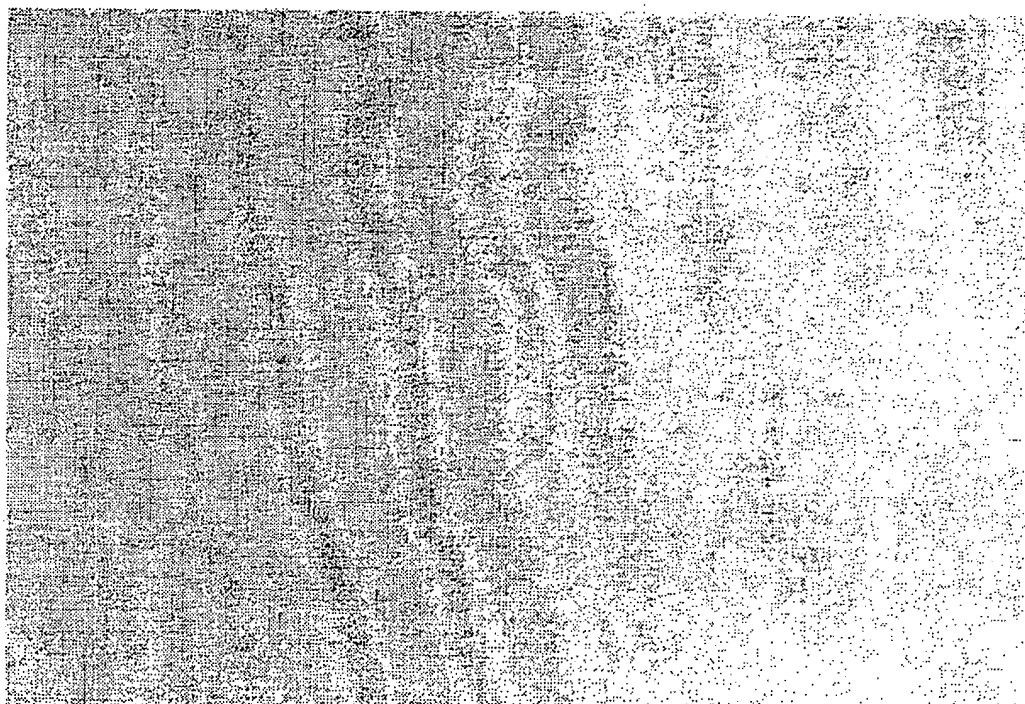


FIGURE 12B

Control 50X

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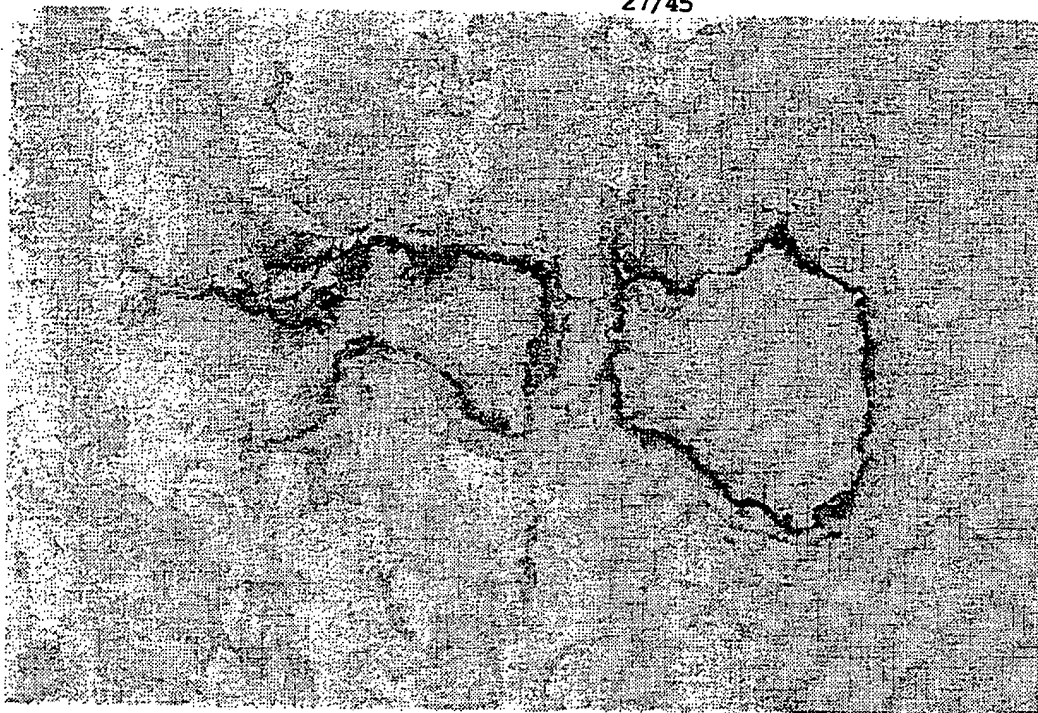


FIGURE 12C

100X

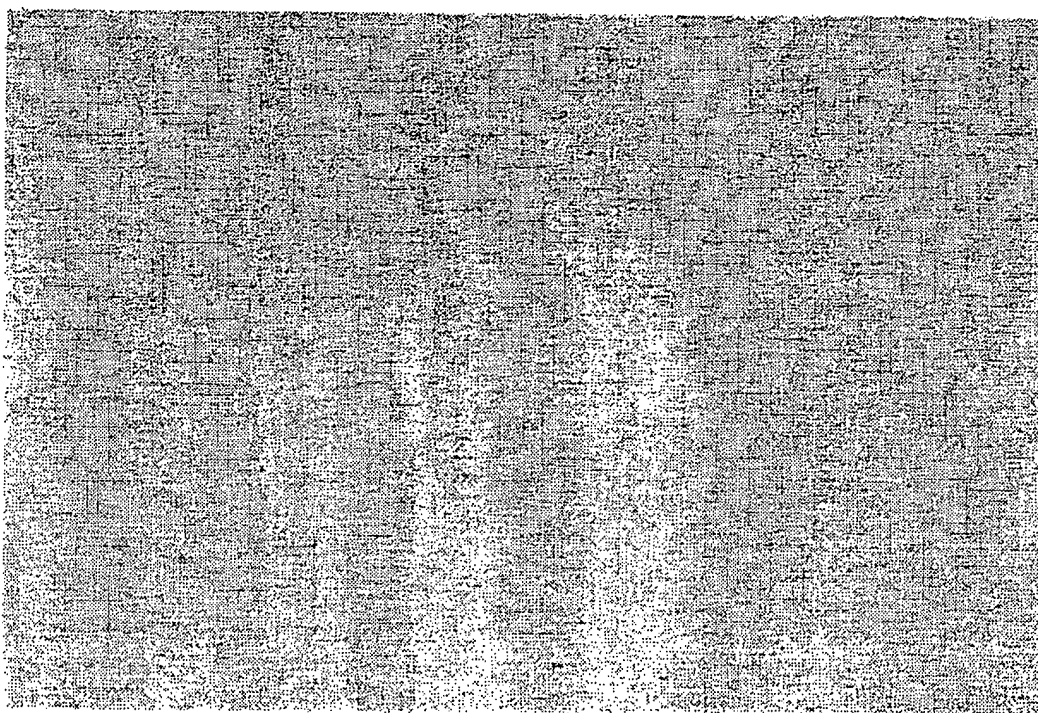


FIGURE 12D

Control 100X

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FIGURE 12E

250X

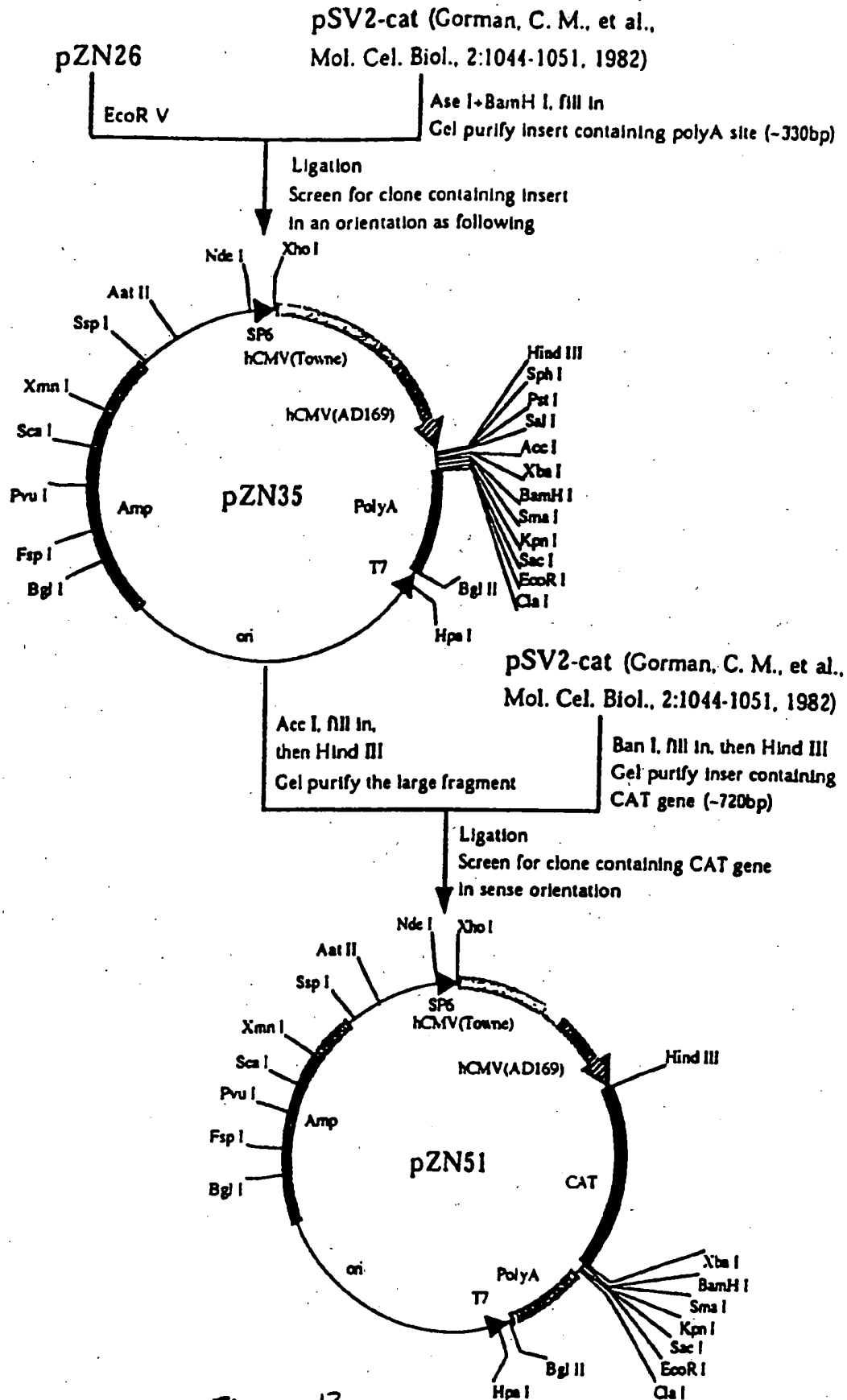


Figure 13  
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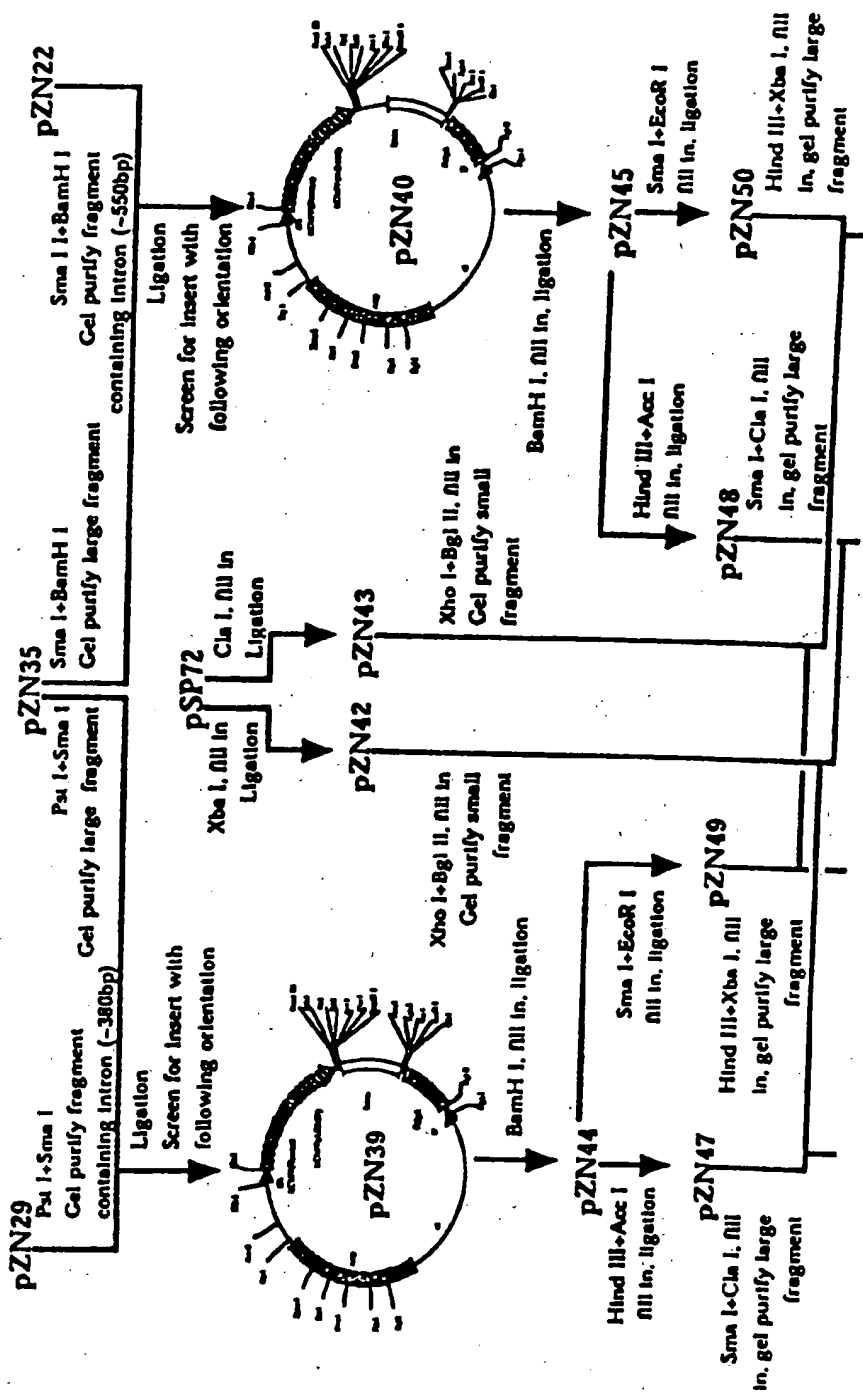


Figure 14

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Figure 14A

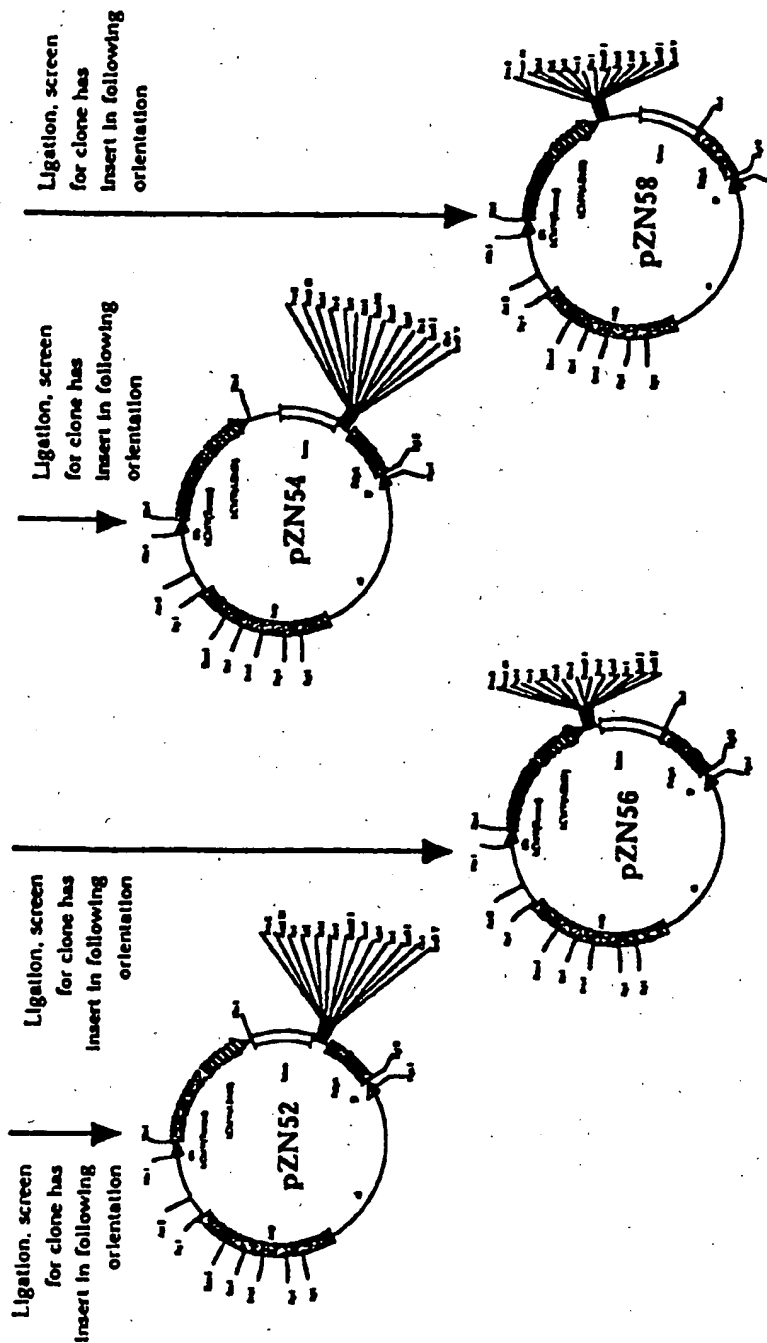


Figure 14B

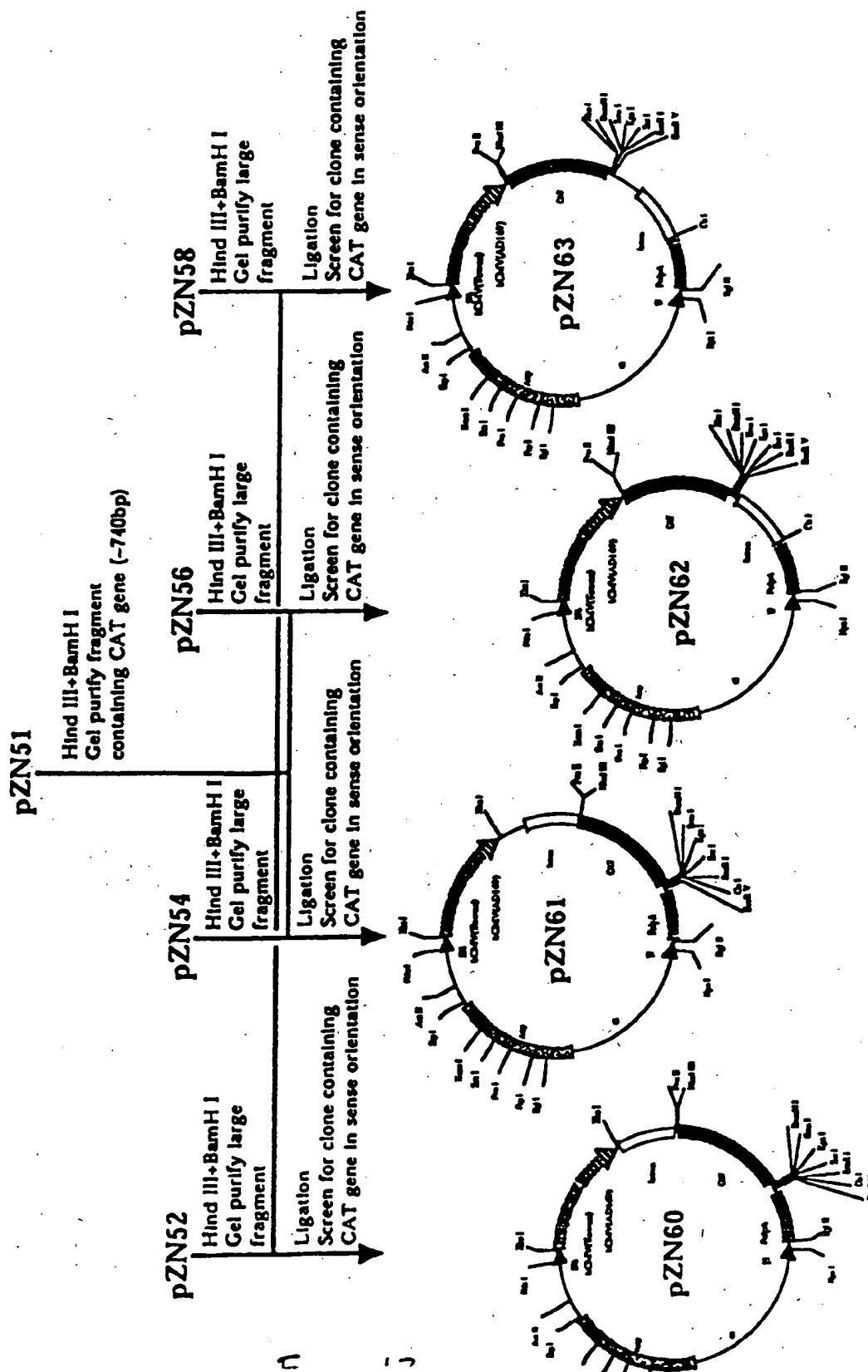


Figure 14C

CAT EXPRESSION WITH DIFFERENT PLASMIDS COMPLEXED TO LIPID CARRIERS

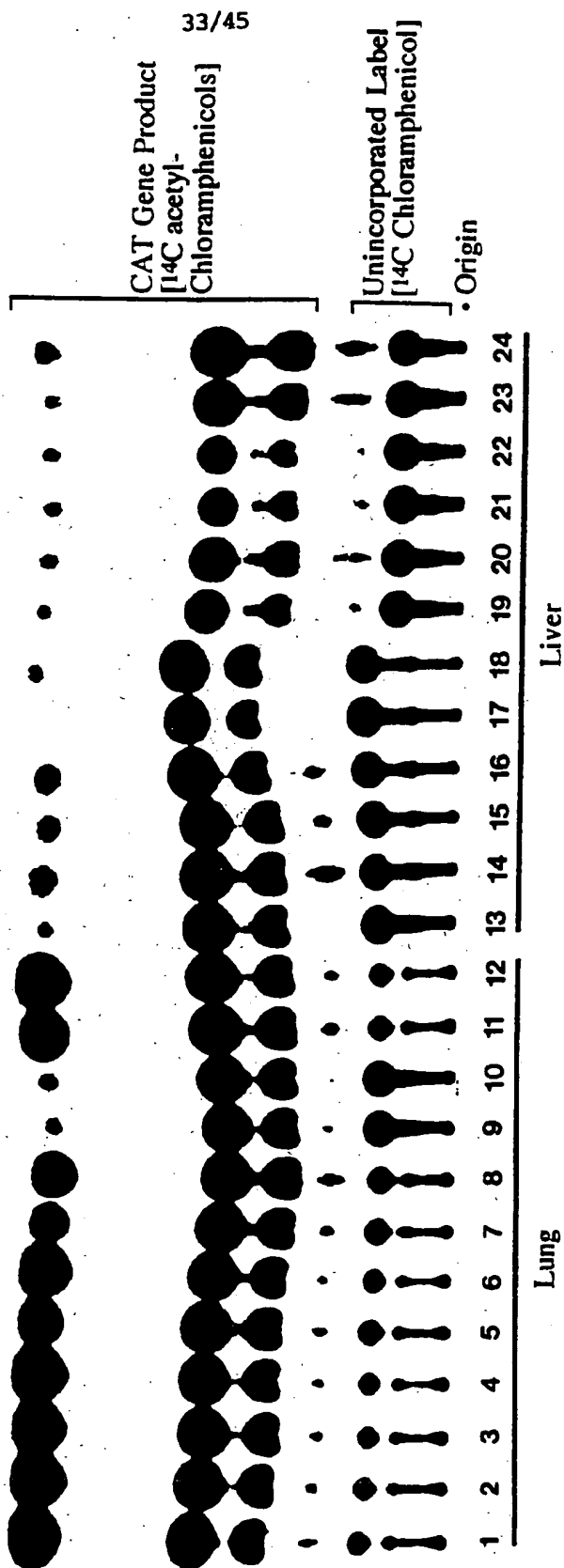


FIGURE 15  
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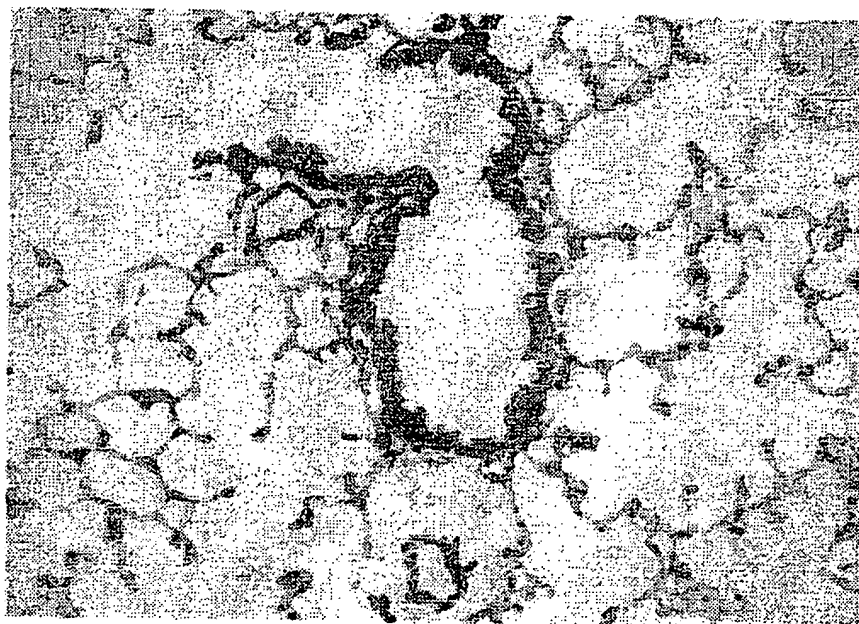


FIGURE 16A

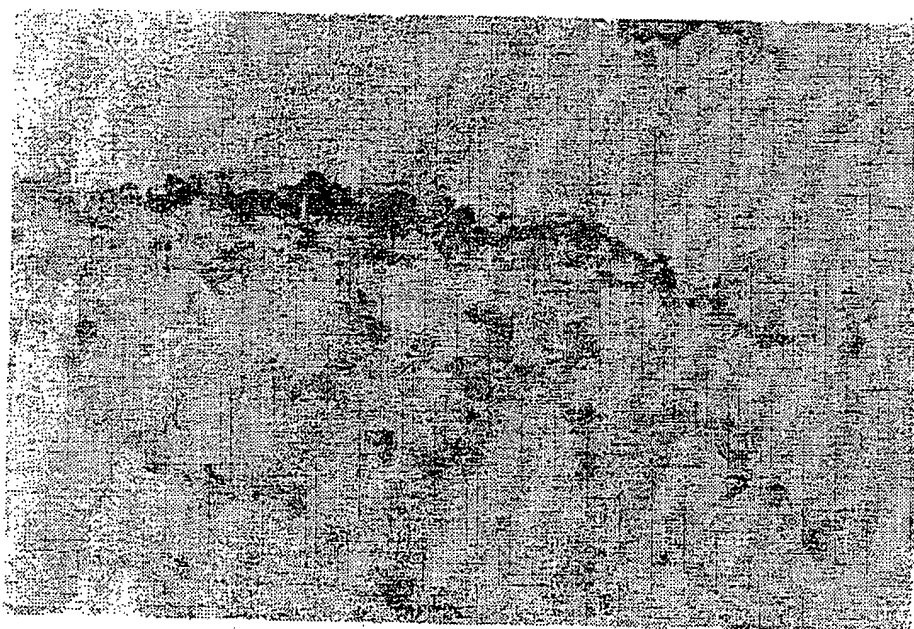


FIGURE 16B

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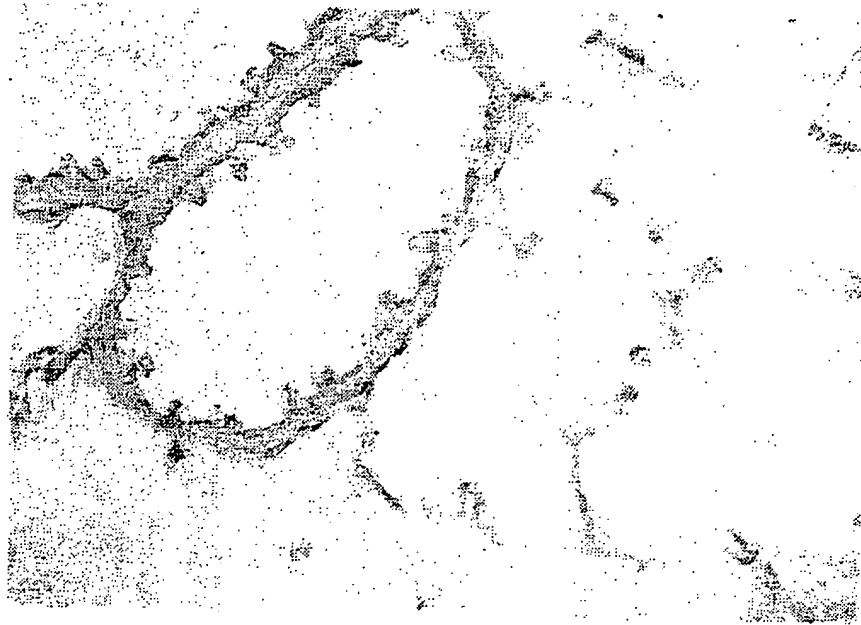


FIGURE 16C

Control

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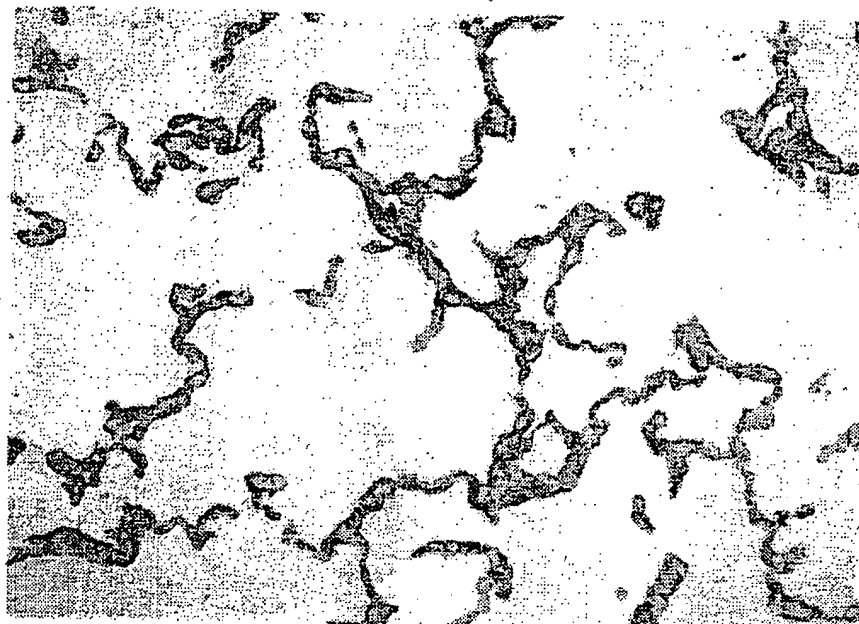


FIGURE 16D

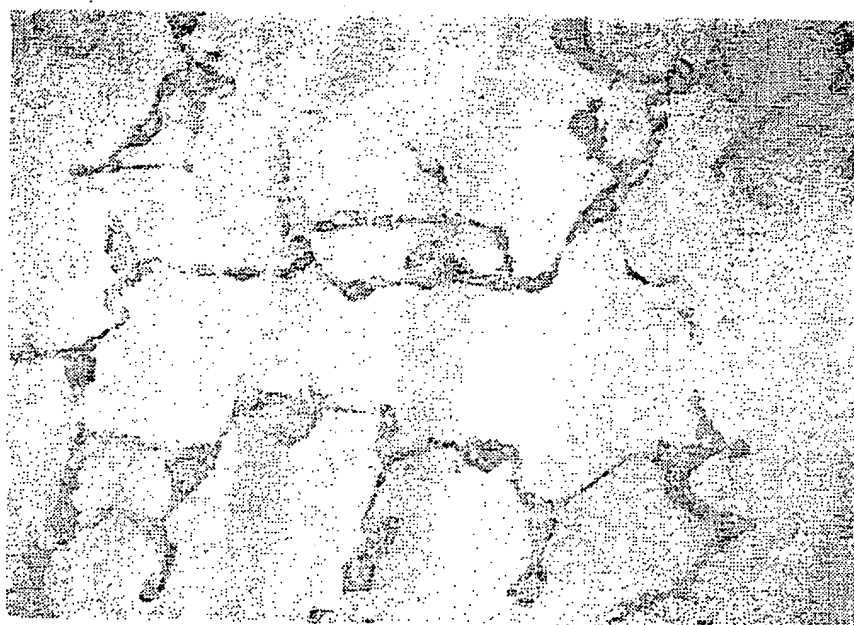
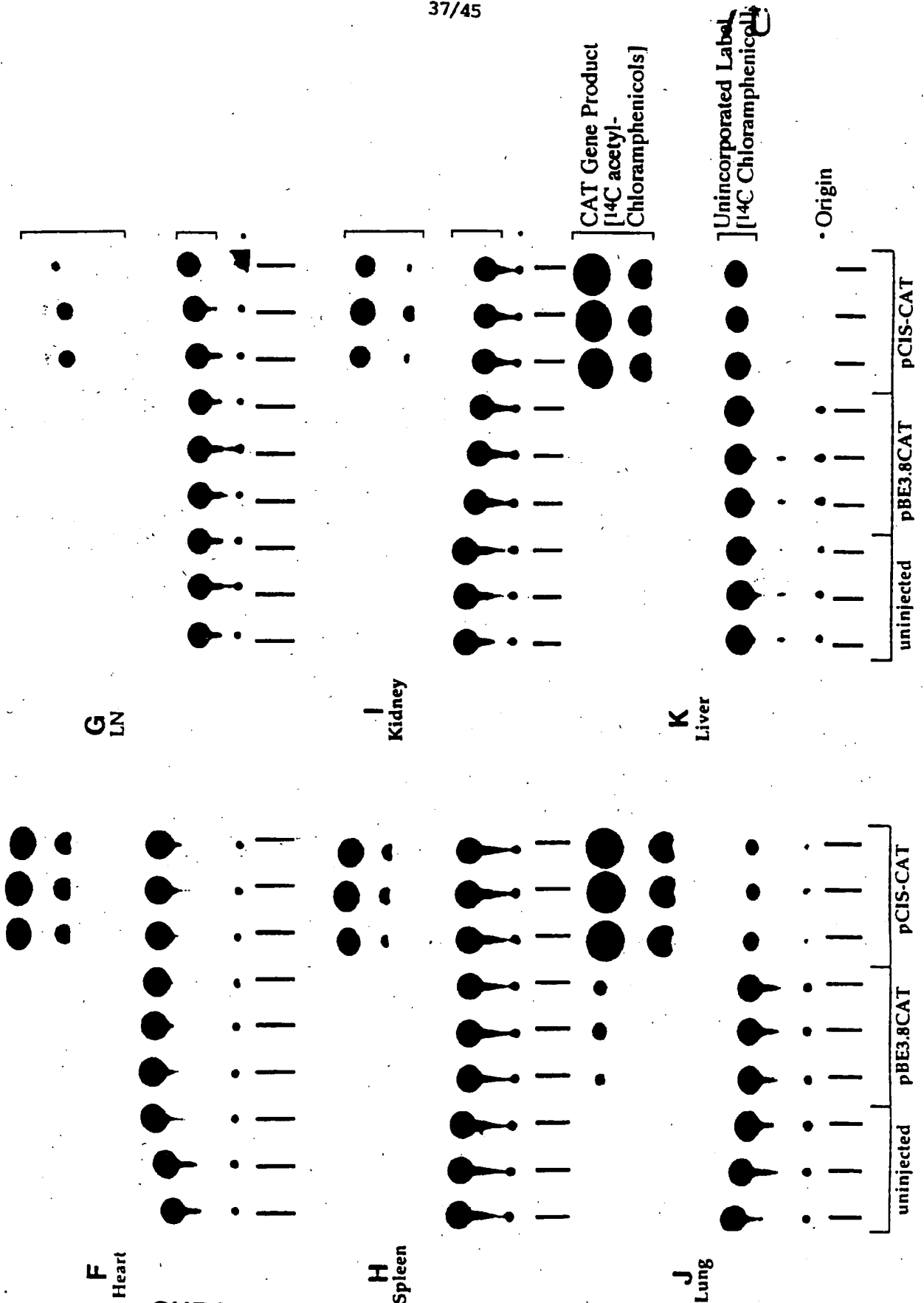
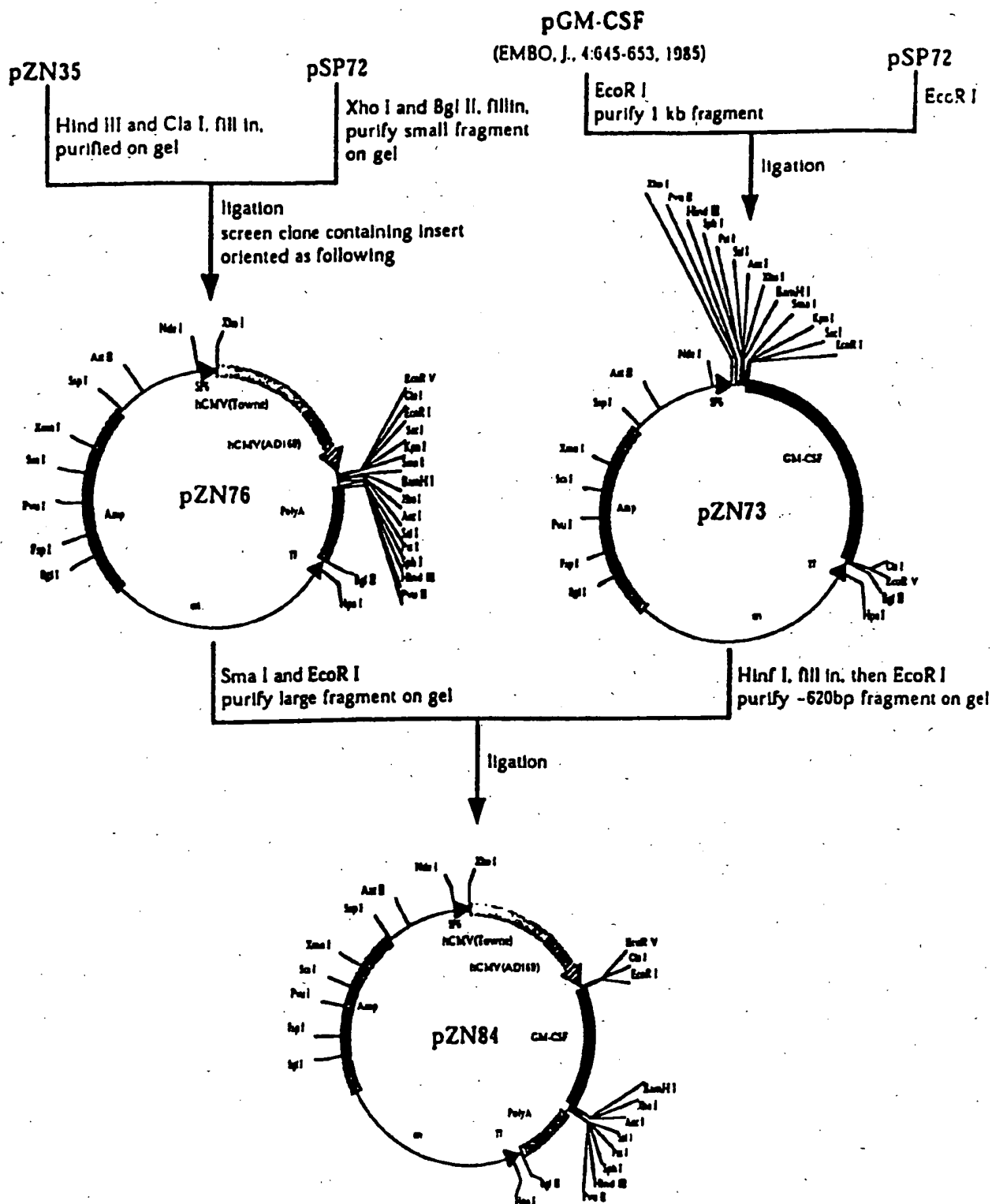


FIGURE 16E



SUBSTITUTE SHEET FIGURE 16(F-K)



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pBQ4.7 (CFTR cDNA clone, from Dr. Francis Collins)

pZN76

Xma I and Sal I,  
gel purify the large fragment

Xma I and Sal I

Ligation

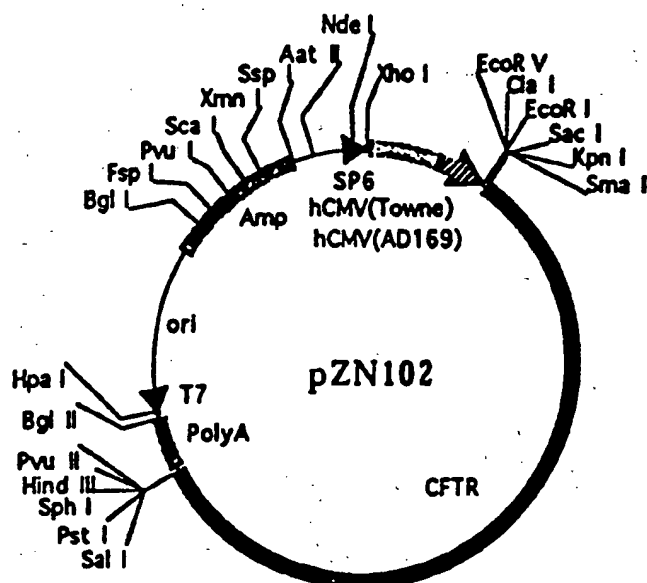


Figure 18

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CAT EXPRESSION WITH pZN27 ALONE

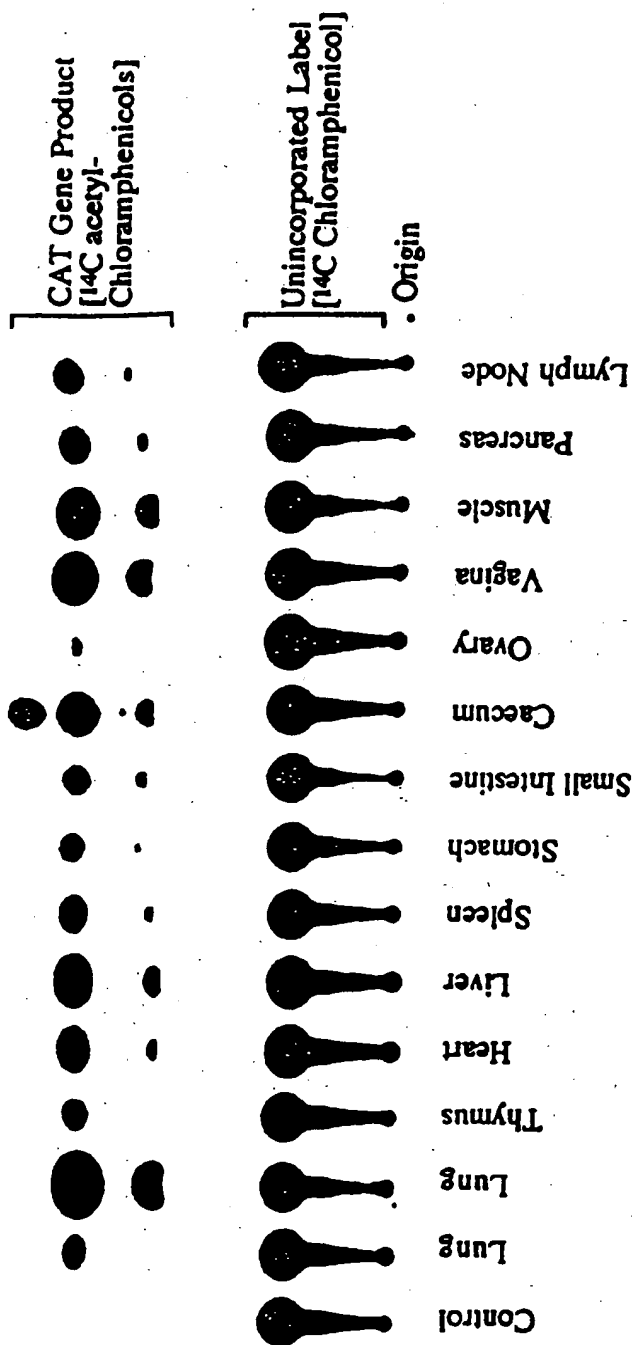


FIGURE 19A

CAT EXPRESSION WITH pZN27 COMPLEXED TO A LIPID CARRIER

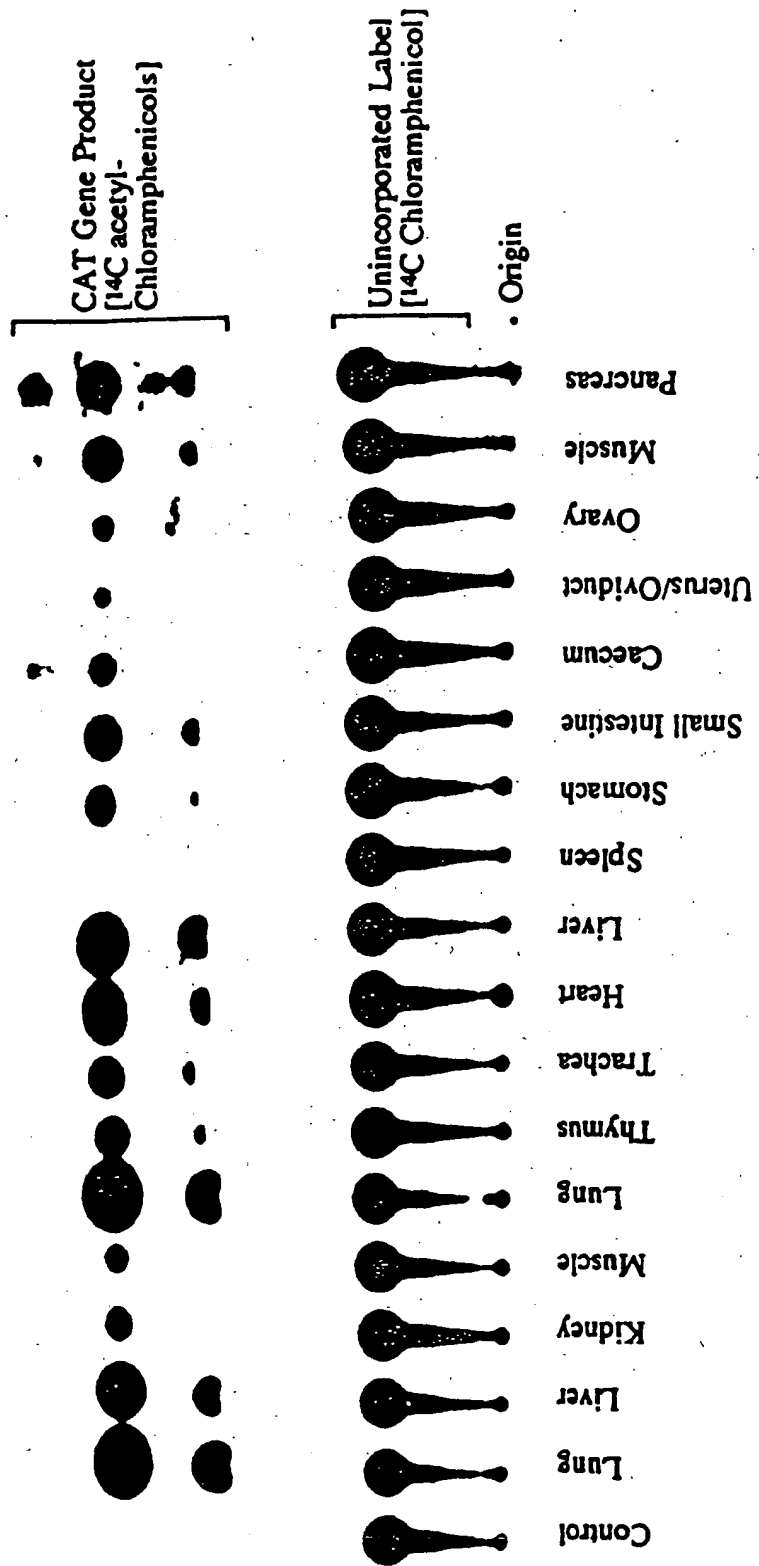


FIGURE 19B  
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CAT EXPRESSION AFTER INJECTION OF PLASMID INTO MOUSE BRAIN RIGHT VENTRICLES

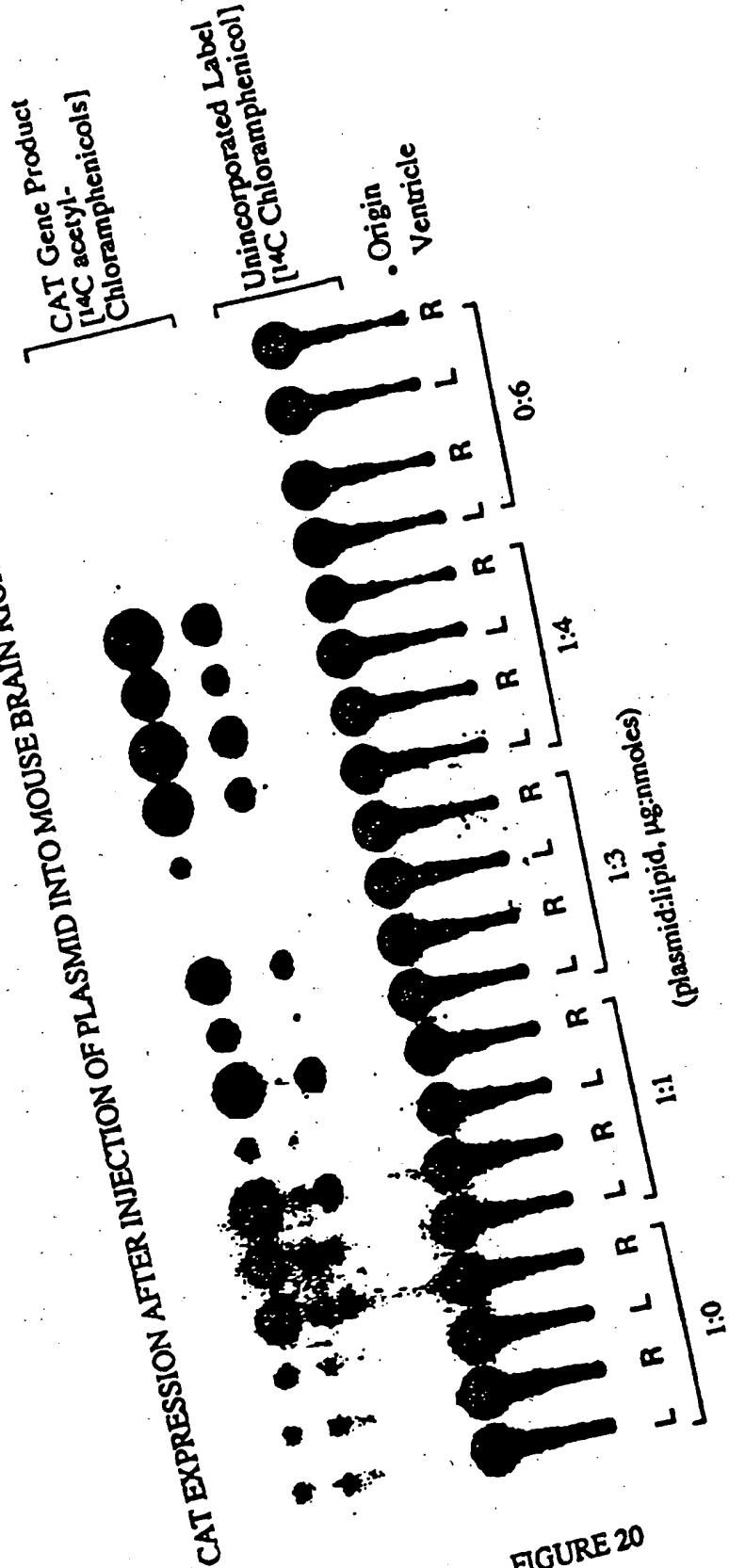


FIGURE 20  
SUBSTITUTE SHEET

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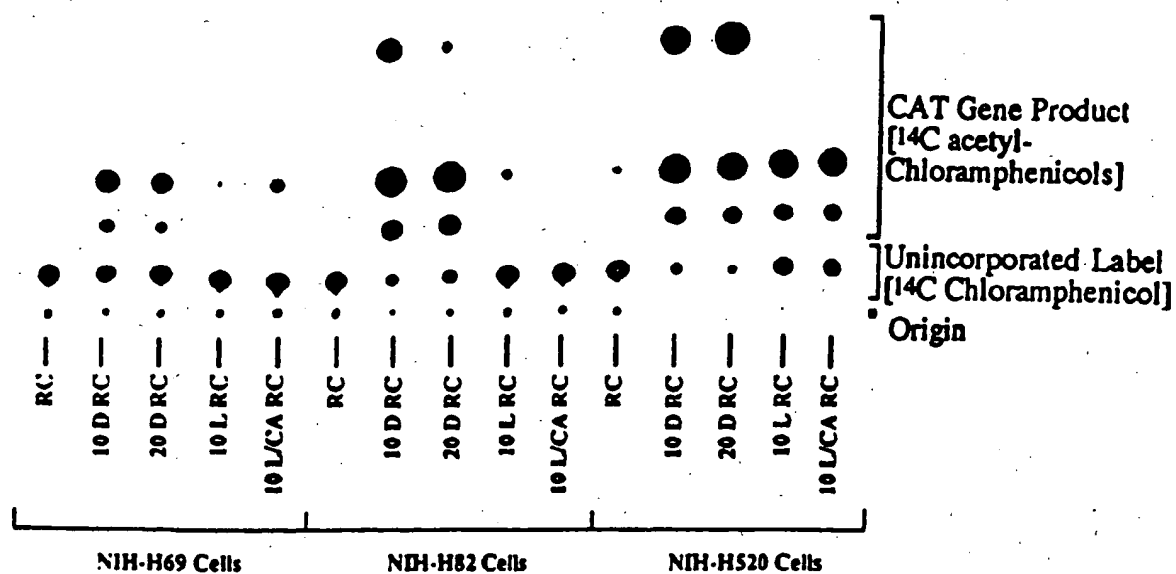
CAT EXPRESSION AFTER TRANSFECTION  
OF HUMAN LUNG CANCER CELL LINES

FIGURE 21

**IMMUNOREACTIVE GM-CSF**  
(Expressed relative to pre-inoculation value)<sup>7</sup>

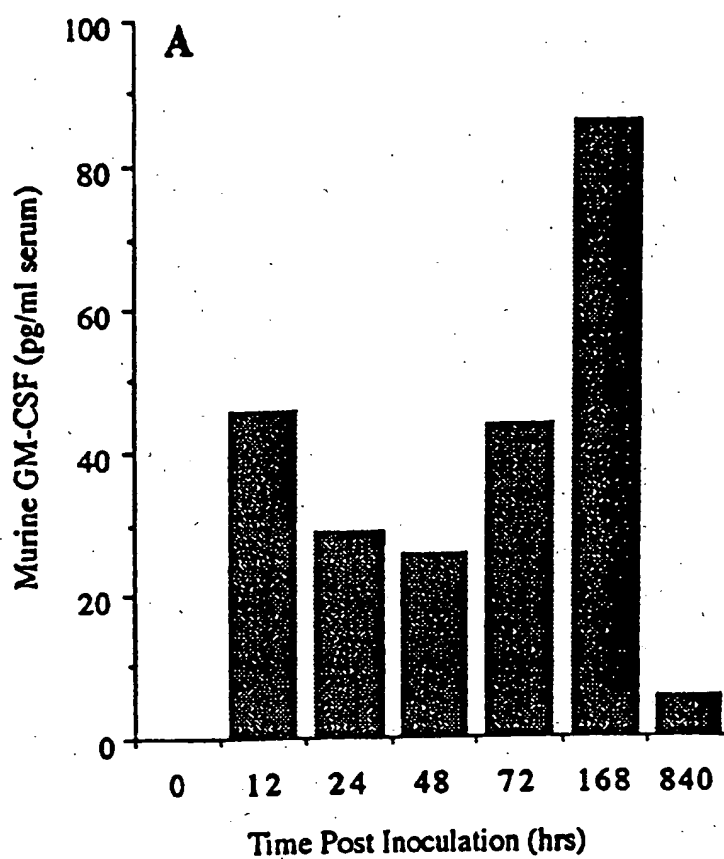


FIGURE 22

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IMMUNOREACTIVE GM-CSF  
(Expressed relative to pre-inoculation value)

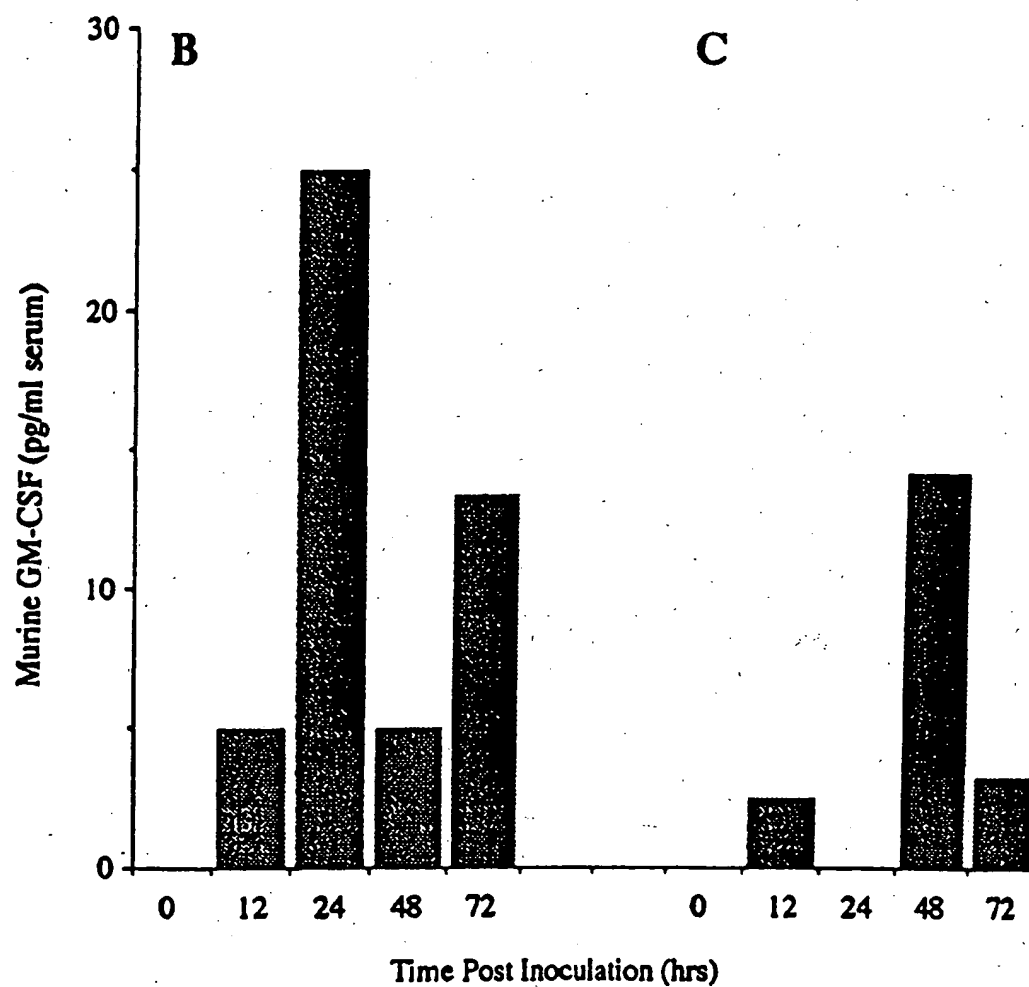


FIGURE 23

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>5</sup> : C12N 15/00, A61K 48/00, A01K 67/027 C12N 15/12, 15/88, 15/11, 15/26, 15/27 A61K 31/70		A3	(11) International Publication Number: <b>WO 93/24640</b>  (43) International Publication Date: 9 December 1993 (09.12.93)
(21) International Application Number: PCT/US93/05366  (22) International Filing Date: 4 June 1993 (04.06.93)  (30) Priority data: 07/894,498 4 June 1992 (04.06.92) US 07/927,200 6 August 1992 (06.08.92) US 07/992,687 17 December 1992 (17.12.92) US  (60) Parent Applications or Grants (63) Related by Continuation US 07/894,498 (CIP) Filed on 4 June 1992 (04.06.92) US 07/927,200 (CIP) Filed on 6 August 1992 (06.08.92) US 07/992,687 (CIP) Filed on 17 December 1992 (17.12.92)  (71) Applicant (for all designated States except US): THE RE- GENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 300 Lakeside Drive, 22nd Floor, Oakland, CA- 94612-3550 (US).			(72) Inventors; and (75) Inventors/Applicants (for US only): DEBS, Robert, J. [US/ US]; 12 Eton Way, Mill Valley, CA 94941 (US). ZHU, Ning [US/US]; 2137 17th Avenue, San Francisco, CA 94116 (US).  (74) Agent: RAE-VENTER, Barbara; Fish & Richardson, 2200 Sand Hill Road, Suite 100, Menlo Park, CA 94025 (US).  (81) Designated States: AT, AU, BB, BG, BR, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI pa- tent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>  (88) Date of publication of the international search report: 28 April 1994 (28.04.94)
(54) Title: EXPRESSION CASSETTE WITH REGULATORY REGIONS FUNCTIONAL IN THE MAMMALIAN HOST  (57) Abstract  Novel methods and compositions are provided for introducing a gene capable of modulating the genotype and phenotype into two or more tissues following systemic administration. The gene can be introduced into a mammalian host by way of an ex- pression vector either as naked DNA or associated with lipid carriers, particularly cationic lipid carriers. Multiple individual tis- sues can be transfected using naked DNA. Using a DNA: lipid carrier complex, multiple tissues and cell types can be transfect- ed. The techniques and compositions find use in the palliation or treatment of any of a variety of genetic-based disorders.			

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## INTERNATIONAL SEARCH REPORT

International Application No.  
PCT/US 93/05366

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IPC 5 C12N15/00 A61K48/00 A01K67/027 C12N15/12 C12N15/88  
C12N15/11 C12N15/26 C12N15/27 A61K31/70

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Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E	WO,A,93 12240 (REGENTS OF THE UNIVERSITY OF CALIFORNIA) 24 June 1993 see the whole document ---	1-7, 10-16
X	WO,A,91 02796 (HSC RESEARCH DEVELOPMENT CORPORATION) 7 March 1991 see page 108, line 4 - line 9; claims 1,2,9,19-22,2627,90-97 ---	1,6,10, 11,13-16
X	WO,A,91 06309 (VANDERBILT UNIVERSITY) 16 May 1991 see the whole document ---	1,10-17
X	WO,A,92 05252 (HSC RESEARCH AND DEVELOPMENT) 2 April 1992 see claims; figure 14 ---	1,6
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P,X	WO,A,93 09236 (BAYLOR COLLEGE OF MEDICINE) 13 May 1993 see the whole document ---	1-4, 10-17
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P,X	WO,A,93 04701 (UNIVERSITY OF CONNECTICUT) 18 March 1993 see the whole document ---	8-17
A	WO,A,91 15501 (YALE UNIVERSITY) 17 October 1991 see the whole document -----	1,5



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		EP-A- 0452457	23-10-91
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